Estrogen improves TIMP-MMP balance and collagen distribution in volume-overloaded hearts of ovariectomized females

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Voloshenyuk TG, Gardner JD. Estrogen improves TIMP-MMP balance and collagen distribution in volume-overloaded hearts of ovariectomized females. Am J Physiol Regul Integr Comp Physiol 299: R683–R693, 2010. First published May 26, 2010; doi:10.1152/ajpregu.00162.2010.—Our previous studies demonstrate that 17β-estradiol limits chronic volume overload-induced hypertrophy and improves heart function in ovariectomized rats. One possible cardioprotective mechanism involves the interaction between estrogen, estrogen receptors, and proteins of the extracellular matrix (ECM). The impact of estrogen deficiency and replacement on left ventricular (LV) hypertrophy and ECM protein expression was studied using five female rat groups: intact sham-operated, ovariectomized sham-operated, intact with volume overload, ovariectomized with volume overload, and ovariectomized with volume overload treated with estrogen. After 8 wk, LV protein extracts were evaluated by Western blot analysis for matrix metalloproteinase-2 (MMP-2) and MMP-9, tissue inhibitors of MMPs (TIMP)-1, TIMP-2, TIMP-3, TIMP-4, collagen types I and III, and estrogen receptor α and β expression. MMP proteolytic activity was assessed by zymography. All volume-overloaded groups exhibited LV hypertrophy, which was associated with a loss of interstitial collagen and perivascular fibrosis. After 8 wk of volume overload, 70% of ovariectomized rats developed heart failure, in contrast to only one intact rat. A downregulation of MMP-2, estrogen receptor-α (ERα), and ERβ, and upregulation of MMP-9 and MT1-MMP were found in the volume-overloaded hearts of ovariectomized rats. Estrogen treatment improved TIMP-2/MMP-2 and TIMP-1/MMP-9 protein balance, restored ERα expression, and prevented MMP-9 activation, perivascular collagen accumulation and development of heart failure. However, estrogen did not fully restore ERβ expression and did not prevent the increase of MMP-9 expression or loss of interstitial collagen. These results support that estrogen limits undesirable ECM remodeling and LV dilation, in part, through modulation of ECM protein expression in volume-overloaded hearts of ovariectomized rats.

extracellular matrix remodeling; ovariectomy; gender; hypertrophy; hormone; collagen; MT1-MMP; ventricle

THE RISKS AND BENEFITS ASSOCIATED with estrogen replacement therapy remain uncertain. Conflicting results regarding the effect of estrogen replacement on the outcome of cardiovascular disease have been obtained from population studies, animal models, and clinical trials (2, 16, 20, 21, 32, 33, 61). The inconsistency of these findings demonstrates the lack of understanding of the mechanisms by which estrogen exerts its beneficial effects on the cardiovascular system. Using a rodent model, we demonstrated that hearts of intact female rats are resistant to chronic volume overload-induced adverse cardiac remodeling and dysfunction (12). This apparent cardioprotection is lost following ovariectomy (6). Estrogen replacement delays the development of left ventricular (LV) hypertrophy and dilation, and it prevents the onset of congestive heart failure (CHF) in ovariectomized rats with volume overload (13). However, the mechanisms of estrogen’s protective effects in prevention of adverse myocardial remodeling remain unclear.

Changes in extracellular matrix (ECM) structure are causally associated with alteration of LV function in the progression of heart disease to heart failure (22, 31, 50). Matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) influence LV structural and functional properties, as they determine ECM turnover and remodeling (17). Abnormalities in MMP and TIMP expression, particularly, in MMP-2 and MMP-9, have been described in various cardiovascular diseases (50). However, the influence of ovarian hormone deficiency and estrogen replacement on the MMP and TIMP balance in the volume-overloaded hearts of females has not been studied. Several reports suggest that marked changes in collagen types, rather than the total collagen content, play an important role in determining the structural and functional properties of the heart (40). Increased LV mass and onset of cardiac fibrosis after ovariectomy in aged rat hearts are associated with increased collagen type I/III ratio, as well as decreased expression of both estrogen receptors (ER)-α and β (61). To date, there are no data regarding the influence of ovarian hormone deficiency and estrogen replacement on collagen isoforms, estrogen receptor expression, or TIMP/MMP balance in volume-overloaded hearts. In this study, we found that ovarian hormone depletion exacerbated LV remodeling due to volume overload and was associated with TIMP/MMP imbalance, reduced interstitial collagen, and attenuated estrogen receptor expression. Estrogen treatment prevented the development of heart failure, improved TIMP/MMP balance, and collagen type I/III ratio, and restricted perivascular fibrosis. These estrogenic effects were coupled with partially restored cardiac ER expression in hearts of ovariectomized rats with chronic volume overload.

METHODS

Studies were performed using 8-wk-old female Sprague-Dawley (Harlan Hsd:SD) rats weighing ~200 g at surgery. Rats were housed under standard environmental conditions and maintained on rat chow and tap water ad libitum. Animals were fed Harlan Teklad 2018 rodent diet to minimize the influence of soybean and alfalfa-derived phytoestrogens. All experimental procedures were approved by the Institution’s Animal Care and Use Committee. Anesthesia for surgical procedures was induced with isoflurane (4% induction and 3% maintenance, balance oxygen). A total of five groups of age-matched female rats were studied as follows: intact sham-operated (SHAM), intact with volume overload (VO), ovariectomized sham-operated (SHOX), ovariectomized with volume overload (VOX), and VOX treated with 17β-estradiol (VOX+EST). Five to seven animals were studied in each group (see Table 1 for details).
Surgical procedures. Ovariectomy was performed 5 days prior to fistula surgery. 17β-estradiol, hereafter referred to as estrogen, was administered via subcutaneous time-release pellets (0.25 mg total, 60-day release, Innovative Research of America, Sarasota, FL), which delivered a dose of ~0.02 mg·kg⁻¹·day⁻¹, and were implanted at the time of ovariectomy. As we have previously shown, this dose results in serum estrogen levels comparable to that of intact females (13). Infrarenal aortocaval fistula was created in rats as previously described (12). Briefly, a laparotomy was performed to expose the abdominal aorta and vena cava. An 18-gauge needle was inserted into the aorta below the renal arteries and advanced into the vena cava to create the fistula. At the experimental endpoint, each rat was weighed and anesthetized. Following laparotomy, fistula patency was visually confirmed, and the heart was removed from the chest cavity and placed in ice-cold PBS. The atria and great vessels were removed and the LV (including septum) and right ventricle (RV) were separated placed in ice-cold PBS. The atria and great vessels were removed and the LV (including septum) and right ventricle (RV) were separated and weighed. Lung and uterine weights were also collected; the ovaries were removed prior to weighing.

Experimental endpoints. Rats were studied at 3 days and 8 wk after surgery. In previous studies using male rats, a significant number of ovaries were removed prior to weighing. As we have previously shown, this dose results in serum estrogen levels comparable to that of intact females (13).

Collagen volume fraction. Collagen volume fraction (CVF) was determined in 5-μm midventricular sections of LV stained with collagen-specific picrosirius red, as previously described (14). Interstitial CVF for the entire section was determined by analyzing 20 random regions avoiding perivascular collagen (Nikon Eclipse microscope; Nikon Elements analysis software). Perivascular CVF was determined by analysis of collagen staining of 10 vessels (diameter >100 μm) per ventricular section.

Western blot analysis. Total proteins were extracted from LV tissue and homogenized with protease inhibitor cocktail (Pierce, Rockford, IL). Protein concentrations were determined by Bradford assay (Bio-Rad, Hercules, CA). All samples (10 μg) were run on 10- to 18-well 4–15% Bis-Tris polyacrylamide gel (Bio-Rad) and stained with Coomassie blue to verify equal loading. The proteins were transferred onto nitrocellulose membranes (Amersham, Piscataway, NJ). Ponceau-S staining and actin protein expression were used to confirm successful transfer. The following primary antibodies were used for immunoblotting: ERα (MC-20), MMP-2 (H-76), collagen type I (M-19), and collagen type III (S-17) from Santa Cruz Biotechnology (Santa Cruz, CA); ERβ antibody (2803) from Ana Spec (San Jose, CA); MMP-9 (AB19016), TIMP-1 (AB3300), TIMP-2 (AB107), TIMP-4 (AB816), and MT1-MMP (MMP-14, AB6004) from Chemicon (Temecula, CA); and TIMP-3 (ab66022) from Abcam (Cambridge, MA). Secondary antibody was IgG (Santa Cruz Biotechnology). Other details of immunoreaction and procedures were previously described (13).

Zymography. From each sample, 30 μg of proteins were electrophoresed in nonreducing conditions on an 8% SDS-PAGE gel containing gelatin (1 mg/ml) from porcine skin (Sigma Chemicals, St Louis, MO), as previously described (5, 14).

Data and statistical analysis. Data are presented as means ± SE, with the exception of body and tissue weights, which are expressed as means ± SD. Changes among the five study groups were evaluated by one-way ANOVA. When a significant F ratio (P < 0.05) was obtained, intergroup comparisons were made using a Newman-Keuls post hoc test (39). Statistical significance was taken to be P ≤ 0.05. The immunoblotting data were normalized as a percentage of the average values for the SHAM group. Data processing and statistical analyses were performed using Microsoft Excel and GraphPad analysis software (ver. 5.0, GraphPad Prism, San Diego, CA).

RESULTS

Estrogen attenuated LV hypertrophy. Volume overload induced progressive ventricular hypertrophy (Table 1). At 3 days after surgery, both the intact VO and ovariectomized VOX groups developed a significant increase in LV mass (P < 0.05 vs. SHAM). At this acute time point, uterine weights were significantly reduced in the SHOX and VOX groups; estrogen treatment prevented this decrease. By 8 wk, LV mass was significantly increased in all volume-overloaded groups relative to SHAM and SHOX. No differences were detected in left or right ventricular mass in the VOX or VOX+EST rats compared with the intact VO, indicating a similar degree of ventricular hypertrophy in these groups. However, lung and body weights were both greater in the ovariectomized VOX animals. Estrogen supplementation attenuated the increase of lung weight and reduced body weight in the treated (VOX+EST) group. Uterine weights for the SHOX and VOX groups remained significantly reduced at 8 wk after surgery, with the VOX+EST group not different from SHAM or intact VO.

Estrogen attenuated interstitial collagen disorganization and prevented the development of perivascular fibrosis. The disruption and redistribution of fibrillar collagens in the volume-overloaded heart were found in both intact and ovariectomized rats (Fig. 1). Interstitial CVF was significantly reduced in all volume-overloaded groups, with the greatest decreases occurring in the ovariectomized groups (Fig. 2; P < 0.05 vs. SHAM or intact VOX). Uterine weights for the SHOX and VOX groups remained significantly reduced at 8 wk after surgery, with the VOX+EST group not different from SHAM or intact VO.

Table 1. Group averaged body, LV, RV, lung, and uterine weights at 3 days and 8 wk after surgery

<table>
<thead>
<tr>
<th>Study groups</th>
<th>n</th>
<th>BW, g</th>
<th>LV, mg</th>
<th>RV, mg</th>
<th>Lung, mg</th>
<th>Uterus, mg</th>
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<tr>
<td><strong>Acute 3 days</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>SHAM</td>
<td>5</td>
<td>≥20</td>
<td>483 ± 26</td>
<td>123 ± 18</td>
<td>1038 ± 53</td>
<td>441 ± 180</td>
</tr>
<tr>
<td>SHOX</td>
<td>4</td>
<td>≥197</td>
<td>460 ± 18±</td>
<td>113 ± 5±</td>
<td>1132 ± 50</td>
<td>146 ± 47±</td>
</tr>
<tr>
<td>VO</td>
<td>4</td>
<td>≥193</td>
<td>534 ± 30±</td>
<td>134 ± 26±</td>
<td>1085 ± 30</td>
<td>443 ± 114±</td>
</tr>
<tr>
<td>VOX</td>
<td>4</td>
<td>≥186</td>
<td>529 ± 14±</td>
<td>146 ± 12±</td>
<td>1266 ± 98±</td>
<td>140 ± 34±</td>
</tr>
<tr>
<td>VOX+EST</td>
<td>4</td>
<td>≥172</td>
<td>508 ± 21</td>
<td>146 ± 9±</td>
<td>1169 ± 66</td>
<td>462 ± 56±</td>
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<td><strong>Chronic 8 wk</strong></td>
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<tr>
<td>SHAM</td>
<td>5</td>
<td>≥243</td>
<td>622 ± 25</td>
<td>159 ± 28</td>
<td>1312 ± 80</td>
<td>709 ± 183</td>
</tr>
<tr>
<td>SHOX</td>
<td>5</td>
<td>≥316</td>
<td>733 ± 48±</td>
<td>204 ± 11±</td>
<td>1592 ± 145</td>
<td>67 ± 8±</td>
</tr>
<tr>
<td>VO</td>
<td>7</td>
<td>≥256</td>
<td>1024 ± 297±</td>
<td>324 ± 127±</td>
<td>1887 ± 489</td>
<td>646 ± 123±</td>
</tr>
<tr>
<td>VOX</td>
<td>7</td>
<td>≥322</td>
<td>1140 ± 318±</td>
<td>368 ± 141±</td>
<td>2358 ± 857±</td>
<td>69 ± 7±</td>
</tr>
<tr>
<td>VOX+EST</td>
<td>5</td>
<td>≥206</td>
<td>1008 ± 79±</td>
<td>314 ± 30±</td>
<td>1560 ± 236</td>
<td>682 ± 208±</td>
</tr>
</tbody>
</table>

Reported values are expressed as means ± SD. BW, body weight; LV, left ventricle; RV, right ventricle; SHAM, sham operated; SHOX, ovariectomized sham operated; VO, volume overload; VOX, ovariectomized volume overload; EST, estrogen. Statistical significance denoted as P ≤ 0.05, †versus SHAM; ‡versus SHOX; †‡versusVO; $versusVOX; $versusVOX+EST.
Histologically, the hearts of VOX rats exhibited collagen fragmentation and disorganization. The decrease of interstitial CVF was not prevented by estrogen replacement; however, estrogen treatment attenuated collagen disorganization with an interstitial distribution of collagen comparable to that of intact VO rats. Volume overload induced a significant increase in perivascular collagen in both the intact VO and ovariectomized VOX rats \((P < 0.05 \text{ vs. SHAM})\). In contrast to the changes in interstitial collagen distribution, perivascular fibrosis was prevented by estrogen treatment.

**Estrogen attenuated the decrease of collagen type I/III ratio.** To better understand the mechanism by which estrogen attenuated LV hypertrophy and dilation, we evaluated collagen-type expression in our chronic study groups. Ovariectomy led to a slight, but nonsignificant, increase of collagen type I in the SHOX group (Fig. 3A). Both the intact and ovariectomized volume-overloaded groups, VO and VOX, had significant reductions of collagen I \((P < 0.05 \text{ vs. SHAM})\). Estrogen treatment prevented this decrease of collagen type I, resulting in levels comparable to SHAM and SHOX. Myocardial collagen type III exhibited a different pattern of expression than collagen type I in the study groups (Fig. 3A). The SHOX group had a significant decrease of collagen type III expression vs. SHAM \((P < 0.05)\), while no changes were detected in the VO and VOX groups at 8 wk postsurgery. Estrogen treatment increased LV collagen III expression to a level significantly greater than SHAM or VOX \((P < 0.05)\). The relative optical densities of collagen I (70 kDa) and collagen III (125 kDa) proteins were used to calculate collagen type I/III ratio. The ovariectomized SHOX group exhibited an increase of collagen

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**Fig. 1.** Representative collagen staining of left ventricular sections by picrosirius red (PSR) 8 wk after surgery. All volume-overloaded groups exhibited a loss of interstitial collagen. Perivascular collagen was markedly increased in the ovariectomized VOX group. Estrogen treatment prevented the accumulation of perivascular collagen, but it did not restore interstitial collagen (SHAM, sham operated; VO, intact volume overloaded; VOX, ovariectomized volume overloaded; EST, estrogen).
As such, ECM-degrading capacity is determined by the ratio of role in preventing excessive ECM degradation by MMPs (29). The expression of MMP-2 in the VO group was significantly reduced compared with the intact VO after 3 days and 8 wk of volume overload. This decrease was prevented by estrogen treatment. MMP-2 protein levels in the VOX group were similar to those found in the intact VO. Volume overload was associated with increased TIMP-2 expression and TIMP-2/MMP-2 ratio at 3 days and 8 wk, compared with the SHAM and SHOX groups (Figs. 5 and 6). At 8 wk, estrogen significantly attenuated the TIMP-2 overexpression and increase of TIMP-2/MMP-2 ratio relative to the VOX group (P < 0.05), but the values remained higher than SHAM.

Estrogen restored the increase of TIMP-1/MMP-9 ratio in response to volume overload. Western blot analyses revealed a significant elevation of MMP-9 and TIMP-1 protein expression in the acute and chronic VO, VOX, and VOX + EST groups (Figs. 7A and 8A). In the chronic VOX group, the increase of TIMP-1 protein in response to volume overload was attenuated, compared with VO (Fig. 8A). Estrogen treatment restored TIMP-1 expression to a level comparable to the chronic VO group but failed to prevent the increase of MMP-9 expression. Calculation of TIMP-1/MMP-9 ratios demonstrated different trends in the acute and chronic groups. Acute volume overload led to increased TIMP-1/MMP-9 in all groups (Fig. 7B). The increase of TIMP-1/MMP-9 was less in the VOX group than in the intact VO group and was restored to VO level by estrogen treatment. Chronically, there was no significant difference in the TIMP-1/MMP-9 ratio between the intact VO group and SHAM (Fig. 8B). However, this ratio was significantly decreased in the ovariectomized VOX group (P < 0.05 vs. SHAM). Estrogen treatment prevented this decrease in cardiac TIMP-1/MMP-9 ratio and resulted in a value comparable to that found in the VO and SHAM groups.

Estrogen treatment had no effect on ventricular TIMP-4/MT-1MMP ratio. Among the four members of the TIMP family, TIMP-4 is most abundant in the myocardium and has been implicated in myocardial dysfunction. TIMP-4 binds to MT1-MMP, inhibiting its autocalytic deactivation and blocking its ability to activate MMP-2 (19, 55). MT1-MMP was expressed at very low levels in the SHAM and SHOX groups (Fig. 9A) and was significantly increased in response to chronic volume overload in all groups (P < 0.05 vs. SHAM). The ovariectomized VOX group exhibited a marked elevation of MT1-MMP expression compared with untreated VOX, which was not different from the intact VO group; yet, the ratio of collagen I/III was not restored to SHAM values (P < 0.05).

Estrogen attenuated cardiac MMP-9 activation, but not MMP-2. Volume overload-induced hypertrophy was associated with increased MMP-2 activity at 8 wk postsurgery; however, MMP-9 activity was only increased in the VOX group (Fig. 4). Estrogen replacement completely blocked the elevation of MMP-9 activity, but it did not prevent MMP-2 activation. No significant differences in MMP-2 or MMP-9 activities were detected in the ovariectomized SHOX group vs. SHAM.

Estrogen attenuated the increase of TIMP-2/MMP-2 ratio in acute and chronic volume overload. TIMPs play an important role in preventing excessive ECM degradation by MMPs (29). As such, ECM-degrading capacity is determined by the ratio of MMP and TIMP secretion. We have calculated TIMP/MMP ratios on the basis of in vitro evidence of TIMP substrate specificity, demonstrating that TIMP-1 forms a preferential complex with pro-MMP-9, whereas TIMP-2 binds to pro-MMP-2 (58). The highest level of pro-MMP-2 protein expression was found in the VO and VOX + EST groups at the acute and chronic time points (Figs. 5A and 6A; P < 0.05 vs. SHAM). The expression of MMP-2 in the VOX group was significantly reduced compared with the intact VO after 3 days and 8 wk of volume overload. This decrease was prevented by estrogen treatment. MMP-2 protein levels in the VOX + EST group were similar to those found in the intact VO. Volume overload was associated with increased TIMP-2 expression and TIMP-2/MMP-2 ratio at 3 days and 8 wk, compared with the SHAM and SHOX groups (Figs. 5 and 6). At 8 wk, estrogen significantly attenuated the TIMP-2 overexpression and increase of TIMP-2/MMP-2 ratio relative to the VOX group (P < 0.05), but the values remained higher than SHAM.

**Fig. 2.** Collagen volume fraction (CVF) calculated from PSR-stained ventricular sections at 8 wk after surgery. A: interstitial CVF was significantly reduced in all volume overloaded (VO and VOX) groups relative to SHAM. Estrogen (EST) did not prevent this reduction in CVF. B: perivascular CVF was increased by volume overload (VO), but it was significantly attenuated by estrogen treatment (P ≤ 0.05, *vs. SHAM; &vs. VO; &vs. VOX; bvs. VOX + EST; **P ≤ 0.01).
Estrogen increased ventricular estrogen receptor expression in ovariectomized rats. Volume overload induced an increase of LV ERβ protein expression in the intact VO group but had no effect on ERα (Fig. 10). In the ovariectomized VOX group, this elevation of ERβ expression was lost, and ERα protein was significantly decreased ($P < 0.05$ vs. VO). Estrogen treatment increased cardiac ERα expression and partially restored the ERβ response to volume overload ($P < 0.05$ vs. VOX). Although ERβ protein was significantly greater in the VOX+EST group than SHAM, expression was not completely restored to the level found in the intact VO group.

Estrogen prevented the progression to heart failure in ovariectomized rats. At 8 wk postfistula, only one intact female (14%) developed congestive heart failure vs. five (70%) of the ovariectomized females. None of the estrogen-treated animals developed CHF. These findings agree with what we have previously reported in a functional assessment of similar treatment groups (13). The severity of disease progression was greatest in the ovariectomized females and...
DISCUSSION

We have previously demonstrated the cardioprotective effects of estrogen treatment against chronic volume overload-induced ventricular remodeling in both male and ovariectomized female rats (13, 14). In those studies, estrogen treatment attenuated LV hypertrophy and dilatation caused by chronic volume overload, which resulted in an improvement of diastolic and systolic function. The goal of our current study was to investigate the ECM-related mechanisms involved in this estrogenic cardioprotection by evaluating the influence of estrogen treatment on MMP, TIMP, and collagen profiles in the volume-overloaded female rat heart. To our knowledge, no other study has examined the influence of ovarian hormone deficiency and estrogen supplementation on collagen and TIMP/MMP balance in volume-overloaded female hearts. The most significant findings of our investigation were that estrogen-dependent cardioprotection was associated with improved ventricular TIMP/MMP balance, collagen distribution, and partial estrogen receptor restoration.

Volume overload alters ventricular collagen distribution. Consistent with our previous findings, volume overload induced progressive ventricular remodeling, including significant LV and RV hypertrophy by 8 wk postfistula (13). Although there were no significant differences in hypertrophy between intact and ovariectomized females, our histological data demonstrated that the spatial distribution of collagen in these hearts exhibited prominent dissimilarity. The greatest alteration in collagen distribution was found in the VOX group, suggesting that the loss of interstitial collagen coupled with increased perivascular collagen contributes to LV dilatation. These findings of reduced interstitial collagen with increased perivascular fibrosis are consistent with previous studies in male rats (22). Our results demonstrating the prevention of perivascular fibrosis by estrogen treatment in volume overload are in agreement with studies by others in profibrotic models of cardiovascular disease, where estrogen reduced perivascular collagen accumulation (45, 46). However, estrogen supplementation did not attenuate the loss of interstitial collagen. These findings may explain, in part, why estrogen does not fully restore systolic function in ovariectomized rats with chronic volume overload and suggest that additional ovarian hormones play a role in supporting the interstitial collagen matrix (13).

Fig. 4. MMP-2 and -9 activities in LV extracts as determined by gelatin zymography at 8 wk after surgery (n = 5 or 6 per group). MMP-2 activity increased in both the intact VO and ovariectomized VOX groups, while MMP-9 activity was significantly elevated only in the VOX group. Estrogen prevented MMP-9 activation but had little effect on MMP-2 activity (P ≤ 0.05, *vs. SHAM; †vs. SHOX; ‡vs. VO; §vs. VOX; and ‡vs. VOX+EST).

Fig. 5. Left ventricular MMP-2 and TIMP-2 expression in response to acute volume overload. For all Western blot analyses of acute (3 day) study groups, n = 4 or 5. A: by 3 days after surgery, MMP-2 expression was significantly reduced in the ovariectomized VOX group relative to SHAM and intact VO. TIMP-2 expression was increased in all volume-overloaded groups. Estrogen treatment prevented the decrease of MMP-2 protein, restoring expression to levels found in SHAM, but had no effect on TIMP-2. B: TIMP-2/MMP-2 ratio was significantly increased by volume overload and was greatest in the ovariectomized VOX group. Estrogen attenuated this increase, returning TIMP-2/MMP-2 to values comparable to intact VO (P ≤ 0.05, *vs. SHAM; †vs. SHOX; ‡vs. VO; ‡vs. VOX; and ‡vs. VOX+EST).
Collagen type I and III expression. The changes in spatial collagen distribution in volume-overloaded female rat hearts were associated with alterations of collagen expression. The decrease of collagen type I in the intact VO and ovariectomized VOX groups occurred concurrently with an increase of MMP expression and activity. This loss of collagen peptides is in accordance with the observed histological decrease and disruption of the fine interstitial collagen network in the volume-overloaded groups. The greatest decrease of interstitial collagen staining was found in the hearts of VOX rats, which corresponded to the lowest protein expression of collagen type I, the highest expression of MMP-9, and the greatest activation of MMP-2 and MMP-9 of all the groups. These results suggest that the loss of collagen type I peptides, due to enhanced MMP activity, are a possible underlying mechanism of the advanced LV remodeling in ovariectomized vs. intact rats subjected to chronic volume overload (13). Our findings are similar to data reported by Iimoto et al. (23), who also concluded that a decrease of collagen type I was associated with LV dilation in the volume over loaded canine heart. Notably, estrogen treatment prevented the loss of collagen I peptides, and increased collagen type III expression, which cumulatively shifted the collagen type I/III ratio to a value not different than the intact VO group. Few studies have examined the effect of estrogen on collagen type III expression. The increase of collagen type III in response to estrogen administration was found in hearts of aged and Dahl salt-sensitive rats (8, 61). Similar estrogenic effects on collagen III were demonstrated in the arteries and other tissues of ovariectomized animals (7, 10, 15, 24, 36, 47). The mechanisms of enhanced collagen type III expression due to estrogen treatment are still unknown, but they are likely related to the changes in MMPs and TIMPs expression that we
identified in this study. These alterations in the proportion of collagen type III may also be caused by increased collagen type III turnover or synthesis, which could reflect enhanced repair of disordered myocardial collagen. Indeed, Meyer et al. (37) reported the association of increased collagen type III turnover with rapid wound healing and tissue repair. Given the substantial loss of interstitial collagen observed in volume overload, we conclude that the increase of collagen type III expression in response to estrogen was beneficial. Additional studies are needed to clarify the mechanisms responsible for these collagen changes. Taken together, our data and these previous reports indicate that estrogen treatment attenuates cardiac function, at least in part, by modulating the relative proportion of collagen types. The improvement of collagen type I/III ratio has clinical relevance, as altered collagen proportion was associated with depressed cardiac function in patients with dilated cardiomyopathy, coronary artery disease, and with progression to heart failure in animal models of cardiac disease (4, 35, 43, 44, 59, 60, 62).

Ventricular TIMP/MMP balance. Accompanied by the changes in collagen spatial distribution were alterations in ventricular MMP and TIMP expression. Concomitant with the decreased interstitial collagen, we found the increase of TIMP-2 expression and TIMP-2/MMP-2 ratio in all volume-overloaded groups at the acute and chronic time points. The decline of MMP-2 protein expression led to the increase of the TIMP-2/MMP-2 ratio in the VOX group. Decreased MMP-2 protein expression suggests impaired catabolism and decreased rate of ECM turnover (5, 51). In agreement with previous studies, we found that estrogen supplementation prevented the downregulation of MMP-2 in the acute and chronic stages of LV remodeling (8, 61). Similar estrogenic effects on MMP-2 expression were found in the ventricle and arteries of ovariectomized rats (27, 61, 64), rat uterus (48), and in cell culture studies (9, 18). Estrogen also has a direct regulatory effect on MMP-2 expression in cardiac fibroblasts and vascular smooth muscle cells (18, 34, 53). In progressive heart disease, modest increases of TIMP-2/MMP-2 ratio are associated with compensatory hypertrophy (56), whereas higher ratios correspond to heart failure and dilated cardiomyopathy (1, 52, 54, 57).

Fig. 8. Left ventricular MMP-9 and TIMP-1 protein expression in response to chronic volume overload. A: MMP-9 and TIMP-1 expression were increased in all volume-overloaded groups at 8 wk after surgery. The greatest increase of MMP-9 expression occurred in the ovariectomized VOX group and was not prevented by estrogen treatment. Although increased by volume overload, TIMP-1 expression was attenuated by ovariectomy in the VOX group, and expression was restored by estrogen treatment. B: unlike the acute groups, the TIMP-1/MMP-9 ratio was decreased by volume overload, with the greatest decrease occurring in the VOX group. Estrogen largely attenuated this decrease resulting in a ratio similar to the intact VO group (P ≤ 0.05, * vs. SHAM; # vs. SHOX; # vs. VO; & vs. VOX; # vs. VOX + EST).

Fig. 9. Left ventricular MT1-MMP (MMP-14) and TIMP-4 protein expression in response to chronic volume overload. A: significant elevation of MT1-MMP and TIMP-4 expression were found in all volume overloaded groups at 8 wk after surgery. The VOX group had a dramatic increase in expression of MT1-MMP, which was not significantly altered by estrogen treatment. Estrogen significantly reduced TIMP-4 expression relative to the untreated VOX group. B: TIMP-4/MT1-MMP ratio was significantly decreased in the ovariectomized VOX group, and this decrease was not prevented by estrogen treatment (P ≤ 0.05, * vs. SHAM; # vs. SHOX; # vs. VO; & vs. VOX; # vs. VOX + EST; double symbols denote P ≤ 0.01).
but was not found in the ovariectomized VOX group. Estrogen increased both LV ERα and ERβ expression in the VOX group vs. SHAM. Increased LV ERβ expression was detected in the intact VO group but was not found in the ovariectomized VOX group. Estrogen increased both ERα and ERβ expression in the VOX+EST group vs. VOX but did not fully restore ERβ expression to levels found in the intact VO group (P < 0.05, *vs. SHAM; #vs. SHOX; ¶vs. VO; ¤vs. VOX; ฿vs. VOX+EST).

Taken together, these findings suggest that estrogen treatment led to an improvement of the TIMP-2/MMP-2 ratio, which contributed to the delayed progression of adverse remodeling and heart failure.

Despite the increased TIMP-2 expression, all volume-overloaded groups exhibited enhanced MMP-2 activity, indicating that additional factors contribute to MMP-2 activity. Membrane-bound MT1-MMP can activate MMP-2 via formation of an MT1-MMP-TIMP-2 complex and facilitates ECM degradation (28, 42, 58). Additionally, TIMP-4 acts as an important regulator of MMP-2 and MT1-MMP activity (3). Although TIMP-4 does not promote MT1-MMP-dependent MMP-2 activation, it does inhibit the auto-degradation of MT1-MMP, which may contribute to increased MT1-MMP expression in the volume-overloaded groups (19, 55). Estrogen treatment attenuated both MT1-MMP and TIMP-4 expression but had little effect on TIMP4/MT1-MMP ratio, which remained significantly lower than that of the intact VO group and may have contributed to enhanced MMP activity. These data, obtained from relatively young adolescent female rats, agree with those of Dai et al. (8), who demonstrated increased cardiac TIMP-4 and MT1-MMP expression in aging hypertensive female rats, suggesting that these proteins may be estrogen dependent.

The results of our study also indicate that estrogen may modulate TIMP-1 and MMP-9 expression. The concurrent elevation of TIMP-1 and MMP-9 expression was found in both intact and ovariectomized rats in response to acute volume overload. However, chronically, TIMP-1 expression was decreased in the VOX group. Our data agree with previous reports that demonstrate the association of decreased TIMP-1 expression and heart failure in humans (29, 54). Similar results were found in hearts of aged ovariectomized rats (30). Further, estrogen treatment restored TIMP-1 expression and TIMP-1/MMP-9 balance in VOX rat hearts, to levels found in intact VO. This restoration of TIMP-1 expression was associated with limited MMP-9 activation; but the volume overload-induced increase of MMP-9 expression was not prevented. These findings indicate that estrogen improved TIMP-1/MMP-9 balance primarily by increasing TIMP-1 expression. However, it is still unclear and warrants further investigation to determine whether these alterations of MMPs and TIMPs expression are caused by a direct estrogenic effect on cardiac cells, or by an indirect systemic influence of estrogen administration.

**Ventricular ERα and ERβ expression.** The important role of ERs in modulating estrogen’s cardioprotective effects has been demonstrated in studies that utilized ER agonists in several animal models, including pressure overload (49), ischemic reperfusion injury (11, 26), and hemorrhage (63). Volume overload caused an increase of LV ERβ expression in the intact rat hearts, while ovariectomy prevented this increase. These results suggest a compensatory protective role of ERβ overexpression in the volume-overloaded heart and are in agreement with data reported from pressure overload and human aortic stenosis (41, 49). Ovarian hormone deficiency was associated with decreased ERα expression, suggesting that a loss of ERα may also contribute to the ECM remodeling during the progression to heart failure in VOX rats (13). Our data and results obtained by others support that reduced ER expression contributes to the progression of cardiac disease and heart failure (13, 61). Estrogen treatment of ovariectomized rats preserved ventricular ERα expression, but the ERβ response to volume overload was only partially restored. These findings suggest that other ovarian hormones may play a role in the regulation of cardiac ERβ expression in response to injury. Concurrent decreases of both ERs appear to have contributed to the adverse myocardial remodeling in the VOX group, but specific roles for each receptor in regulation of the TIMP-MMP balance remains unclear.

**Perspectives and Significance**

We demonstrated the positive influence of estrogen replacement on cardiac ECM protein expression profiles in the acute and chronic stages of volume overload of ovariectomized rats. Estrogen treatment improved ventricular TIMP-MMP balance and collagen type I/III ratio, partially restored cardiac ER expression, blocked MMP-9 activation, and attenuated perivascular fibrosis. These effects were associated with a prevention of volume overload-induced heart failure. Given the influence of estrogen on multiple organ systems and cell types, this cardioprotection was likely produced by a combination of direct and systemic effects of estrogen treatment. However, estrogen replacement had several limitations. Estrogen treatment did not prevent the volume overload-induced expression of MT1-MMP and MMP-9, or the loss of interstitial collagen. Further, estrogen did not restore ventricular ERβ expression in the ovariectomized rat hearts to the level observed in the intact volume-overloaded group. These findings provide novel insight into the ECM mechanisms contributing to estrogen-dependent cardioprotection in the volume overloaded female heart. Additional studies are warranted in aged females, as aging modifies both ECM protein turnover and responsiveness to estrogen. (25, 38, 61).
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