Estrogenic modulation of inflammation-related genes in male rats following volume overload

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McLarty JL, Meléndez GC, Levick SP, Bennett S, Sabo-Attwood T, Brower GL, Janicki JS. Estrogenic modulation of inflammation-related genes in male rats following volume overload. Physiol Genomics 44: 362–373, 2012. First published January 24, 2012; doi:10.1152/physiolgenomics.00146.2011.—Our laboratory has previously reported significant increases of the proinflammatory cytokine TNF-α in male hearts secondary to sustained volume overload. These elevated levels of TNF-α are accompanied by left ventricular (LV) dilatation and cardiac dysfunction. In contrast, estrogen has been shown to protect against this adverse cardiac remodeling in both female and male rats. The purpose of this study was to determine whether estrogen has an effect on inflammation-related genes that contribute to this estrogen-mediated cardioprotection. Myocardial volume overload was induced by aortocaval fistula in 8 wk old male Sprague-Dawley rats (n = 30), and genes of interest were identified using an inflammatory PCR array in Sham, Fistula, and Fistula + Estrogen-treated (0.02 mg/kg per day beginning 2 wk prior to fistula) groups. A total of 55 inflammatory genes were modified (≥2-fold change) at 3 days postfistula. The number of inflammatory gene was reduced to 21 genes by estrogen treatment, whereas 13 genes were comparably modulated in both fistula groups. The most notable were TNF-α, which was downregulated by estrogen, and the TNF-α receptors, which were differentially regulated by estrogen. Specific genes related to arachidonic acid metabolism were downregulated by estrogen, including cyclooxygenase-1 and -2. Finally, gene expression for the β1-integrin cell adhesion subunit was significantly upregulated in the LV of estrogen-treated animals. Protein levels reflected the changes observed at the gene level. These data suggest that estrogen provides its cardioprotective effects, at least in part, via genomic modulation of numerous inflammation-related genes.

MATERIALS AND METHODS

Studies were performed using 8 wk old male (n = 30) Sprague-Dawley (Hsd:SD) rats that were housed under standard environmental conditions and maintained on a commercially available estrogen-free diet (2014 Teklad Global 14% Protein Rodent Maintenance Diet, Harlan Laboratories) and tap water ad libitum. All studies conformed to the principles of the APS Guiding Principles in the Care and Use of Vertebrate Animals in Research and Training and were approved by the University of South Carolina’s Institutional Animal Care and Use Committee. Surgical anesthesia was induced using isoflurane (4% induction/2.5% maintenance, balance oxygen). Postoperative analgesia was provided by intraoperative administration of buprenorphine hydrochloride (0.025 mg/kg sc). At experimental endpoints, animals were anesthetized with pentobarbital sodium (50 mg/kg ip), and hearts removed under aseptic conditions.

Surgical Procedures

Aorta-caval fistula surgery. An infrarenal aortocaval fistula was created in rats as previously described (5). Briefly, a ventral abdominal laparotomy was performed to expose the abdominal aorta and caudal vena cava. The abdominal aorta and vena cava were occluded proximal and distal to the intended puncture site just below the renal arteries. An 18-gauge needle was inserted into the abdominal aorta and then advanced into the vena cava to create a fistula between the two vessels. The needle was withdrawn, and the ventral puncture site in the abdominal aorta was sealed with surgical glue. The abdominal

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The abdominal aortocaval fistula model of volume overload is an excellent approach in which to study sex differences in heart failure. In this model, males undergo collagen degradation and develop significant left ventricular (LV) wall thinning, ventricular dilatation, and increased myocardial compliance (5, 9, 10), which ultimately progress to congestive heart failure (9). Meanwhile, females develop concentric myocardial remodeling, wherein the LV mass-to-volume ratio increases to maintain normal cardiac function (17). We have shown that this cardioprotection in female rats is modulated by ovarian hormones, chiefly estrogen (8, 18), since following ovariectomy, chronic volume overload induces adverse cardiac remodeling nearly identical to that of males (8). Conversely, estrogen supplementation in ovariectomized female rats protected them from heart failure following chronic volume overload (18). Congruent with this, the cardioprotection produced by estrogen supplementation was also equally effective in males, as reflected by significant attenuation of myocardial remodeling following volume overload, thereby maintaining LV pump function (19). This compelling observation in male rats demonstrates the powerful effects that estrogen can exert. However, the mechanism by which estrogen provides protection against adverse myocardial remodeling and heart failure is unknown. One possibility is that estrogen can modulate proinflammatory pathways in the heart, especially TNF-α (43). Consistent with this, myocardial levels of the proinflammatory cytokine TNF-α are significantly elevated within the first week of chronic volume overload (26, 30, 31). This led us to question whether estrogen may exert its cardioprotective effects by genomic modulation of inflammatory-related components of the remodeling process, including TNF-α. Therefore, we used a PCR array approach and compared inflammatory gene expression in male rat hearts with that from male rats treated with estrogen to identify genes that were modulated by estrogen following induction of volume overload. Estrogen-mediated significant changes in a subset of proinflammatory cytokines, arachidonic acid metabolism-related genes [e.g., cyclooxygenase-1 (COX-1)], and the β1-integrin cell adhesion subunit.
musculature and skin incisions were closed with absorbable suture and autoclips, respectively.

**Estrogen treatment.** Estrogen was delivered via a time release 17β-estradiol pellet (21 day release pellet, Innovative Research of America, Sarasota, FL). Pellets were implanted subcutaneously between the shoulder blades 2 wk prior to fistula surgery. We have previously found that a dose of 0.02 mg/kg per day produces serum levels of ~35 pg/ml, which was comparable to the average estrogen level in intact female rats with functional ovaries (18).

**Experimental Protocol**

Rats were randomly divided into three groups: 1) Sham, 2) Fistula, and 3) Fistula + Estrogen, with n = 10 per group. These groups were studied at 3 days postfistula as we have previously established that TNF-α is significantly elevated at this time-point (26, 30, 31), as well as coinciding with the peak increase in mast cell density and substantial extracellular matrix degradation postfistula (6). It is these initial events that perpetuate the long-term adverse remodeling response (7). At the experimental endpoint, a visual inspection of the fistula for patency was performed. The atria and major vessels were removed from the heart, and the LV, including septum, were separated from the right ventricle (RV) and weighed. Lung weight was measured after the esophagus and trachea were trimmed away and the pleural surface blotted dry.

**PCR Array Analysis**

Following its removal, the LV was prepared for RNA isolation. LV tissue was homogenized in TRizol (Invitrogen, Carlsbad, CA), and chloroform was used to prepare a 2:1 ratio of TRizol to chloroform (n = 4/group). RNA was precipitated with isopropanol then purified using an RNeasy Mini Kit (Qiagen, Germantown, MD) and tested for purity. RNA concentration was determined by optical density at 260 nm. Only samples with an optical density ratio > 1.8 at 260/280 nm were selected. RNA was reversed transcribed to cDNA by PCR using the Promega Reverse Transcription System (Madison, WI). The cDNA was then added to a Taqman Low Density Inflammation Array (Applied Biosystems, Foster City, CA) on a 7900 HT Real-Time PCR System. The complete list of 96 genes assayed on the PCR Array can be found in Supplemental Data Table S1. Relative gene expression data were acquired using real-time quantitative PCR and the 2^-ΔΔCt referenced to 18S rRNA as an internal control for Ct values (29). The gene expression of each individual gene for the Fistula and Fistula + Estrogen groups was normalized to the Sham group. A list of genes with ≥2.0-fold change in expression was determined for the Fistula and Fistula + Estrogen groups, and from this a final list was generated based on significant changes in expression identified by Welch’s t-test with P < 0.05.

**Quantitative Real-Time RT-PCR Amplification**

qRT-PCR was performed on an Applied Biosystems 7900 HT Real-Time PCR System. The primers used were supplied by Applied Biosystems using the Assay Identification and are shown in Supplemental Data Table S1. Briefly, 500 ng of total LV RNA (n = 4/group) was reverse-transcribed in a 20 μl reaction volume using Taqman Gene Expression Assays. Samples were run in duplicate, with 18S rRNA used as an internal control. Twelve genes were randomly chosen to verify the correlation of the qRT-PCR to the Applied Biosystems Inflammation Array in the Fistula and the Fistula + Estrogen groups.

**Western Analysis**

Total protein was extracted from LV tissue (n = 6 per group) by homogenization in buffer containing protease inhibitor cocktail (Pierce, Rockford, IL), and 35 μg of total protein was then loaded on 10% SDS-PAGE gels, separated by electrophoresis, and transferred onto nitrocellulose membranes (Amersham, Piscataway, NJ). Protein concentrations were determined using a Bradford Protein Assay (Bio-Rad Laboratories, Hercules, CA). Ponceau-S staining was used to confirm equal loading and establish transfer of proteins from the SDS-PAGE gel to the nitrocellulose membranes. Membranes were blocked with 5% nonfat milk in TBS-0.01% Tween for 2 h at room temperature.

**TNF-α receptors.** The blots were then incubated either with goat anti-rat TNF receptor I (sc-1070, Santa Cruz Biotechnology, 1:1,000) or rabbit anti-rat TNF receptor II (sc-7862, Santa Cruz Biotechnology, 1:1,000) overnight at 4°C. The blots were then incubated in secondary donkey anti-goat IgG (sc-2020, Santa Cruz Biotechnology, 1:2,000) or secondary goat anti-rabbit IgG conjugated with horseradish peroxidase (sc-2004, Santa Cruz Biotechnology, 1:2,000) overnight at 4°C.

**Cox-1 and Cox-2.** The blots were incubated with either rabbit anti-COX-1 (ab109025, Abcam, 1:100) or rabbit anti-COX2 (ab6665, Abcam, 1:200) for 2 h.

**β1-Integrin.** The blots were incubated with rabbit anti-β1-integrin (sc-8978, Santa Cruz Biotechnology, 1:200) for 2 h. The blots were then incubated in secondary goat anti rabbit IgG (sc-2004, Santa Cruz Biotechnology, 1:3,000). The blots were then probed for GAPDH which served as a loading control (primary antibody: mouse anti-GAPDH, 1:3,000 at room temperature for 2 h, sc-166574, Santa Cruz Biotechnology; secondary antibody: goat anti-mouse IgG, 1:5,000 at room temperature for 2 h, sc-2061, Santa Cruz Biotechnology). Immunoreaction signals were visualized with Pierce enhanced chemiluminescence (Thermo Fisher Scientific, Rockford, IL) by exposure to hyperfilm (Phenix Research Products, Candler, NC). Densitometry analysis was performed with a Bio-Rad GS-800 calibrated densitometer. TNF-α receptor I and II, COX-1 and COX-2, and β1-integrin were quantified as a ratio to GAPDH.

**TNF-α Levels**

Myocardial levels were determined using a commercially available ELISA (R & B Biosystems, Minneapolis, MN). All samples were run in triplicate.

**Prostaglandin Levels**

Myocardial levels of prostaglandin E2 (PGE2) and 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2) were determined using a commercially available ELISA kit from Cayman Chemical (Ann Arbor, MI) and an ELISA kit from EnzoLife Sciences (Plymouth Meeting, PA), respectively. All samples were run in duplicate, with n equal to 6 per group.

**Data and Statistical Analysis**

Grouped data are expressed as means ± SE or means ± SD, where appropriate. Grouped data comparisons were assessed by one-way analysis of variance. When a significant F ratio (P < 0.05) was obtained, intergroup comparisons were made by Bonferroni bounds post hoc test.

### Table 1. Biometric weights of male rats 3 days postfistula surgery

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>BW, g</th>
<th>LV, mg</th>
<th>RV, mg</th>
<th>Lung, mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>10</td>
<td>247 ± 37</td>
<td>740 ± 23</td>
<td>141 ± 30</td>
<td>1,091 ± 47</td>
</tr>
<tr>
<td>Fistula</td>
<td>10</td>
<td>264 ± 12*</td>
<td>787 ± 42*</td>
<td>183 ± 34*</td>
<td>1,282 ± 71*</td>
</tr>
<tr>
<td>Fistula + Estrogen</td>
<td>10</td>
<td>235 ± 3</td>
<td>744 ± 30</td>
<td>154 ± 26</td>
<td>1,251 ± 87*</td>
</tr>
</tbody>
</table>

Values are means ± SD. *P < 0.05 vs. Sham, †P < 0.05 vs. Fistula + Estrogen. BW, body weight; LV, left ventricle; RV, right ventricle.
Table 2. Altered inflammatory genes identified by PCR array in Fistula and Fistula + Estrogen at 3 days postsurgery

<table>
<thead>
<tr>
<th>Accession #</th>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Function</th>
<th>Fistula Value</th>
<th>Estrogen Value</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_012488.2</td>
<td>A2m</td>
<td>alpha 2 macroglobulin</td>
<td>inflammation/stress response</td>
<td>1.04</td>
<td>2.75</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>NM_012701.1</td>
<td>Adrb1</td>
<td>adrenergic receptor, beta 1</td>
<td>sympathetic nervous system</td>
<td>13.23</td>
<td>1.24</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>NM_012492.2</td>
<td>Adrb2</td>
<td>adrenergic receptor, beta 2</td>
<td>sympathetic nervous system</td>
<td>2.01</td>
<td>1.47</td>
<td>0.0022</td>
</tr>
<tr>
<td>NM_012822.1</td>
<td>Alox5</td>
<td>arachidonate 5-lipoxygenase</td>
<td>metabolism</td>
<td>2.27</td>
<td>1.14</td>
<td>0.0003</td>
</tr>
<tr>
<td>NM_012904.1</td>
<td>Anaxa1</td>
<td>annexin A1</td>
<td>membrane scaffolding</td>
<td>3.67</td>
<td>2.25</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>NM_012823.1</td>
<td>Anaxa3</td>
<td>annexin A3</td>
<td>membrane scaffolding</td>
<td>3.06</td>
<td>1.80</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>NM_013132.1</td>
<td>Anaxa5</td>
<td>annexin A5</td>
<td>membrane scaffolding</td>
<td>19.25</td>
<td>1.60</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>NM_012240.2</td>
<td>Arbp</td>
<td>ribosomal protein, large</td>
<td></td>
<td>3.23</td>
<td>1.41</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>NM_012517.2</td>
<td>Cacna1c</td>
<td>calcium channel, voltage-dependent, alpha 1 c subunit</td>
<td>L-Type Calcium Channel</td>
<td>8.04</td>
<td>1.36</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>NM_012798.1</td>
<td>Cacna1d</td>
<td>calcium channel, voltage-dependent, alpha 1 d subunit</td>
<td>L-Type Calcium Channel</td>
<td>171.02</td>
<td>103.17</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>NM_012889.1</td>
<td>Vcam1</td>
<td>vascular cell adhesion molecule 1</td>
<td>membrane adhesion</td>
<td>2.80</td>
<td>9.10</td>
<td>&lt;0.0015</td>
</tr>
</tbody>
</table>

"Fistula" column shows Fistula group PCR values normalized to Sham group; "Fistula + Estrogen" column shows Fistula + Estrogen PCR values normalized to Sham group; "P Value" shows P value of Fistula vs. Fistula + Estrogen.
RESULTS

Biometrics

Average LV, RV, lung, and body weights are listed in Table 1. At 3 days postfistula, there was a significant increase in LV, RV, and lung weights in the Fistula group relative to that in the Sham-operated rats. A significant increase in lung weight also occurred in the Fistula + Estrogen group compared with the Sham-operated rats. In contrast, the LV and RV weights in the Fistula + Estrogen

Fig. 1. Distribution of changes in left ventricular gene expression as determined by PCR array analysis and subsequent validation by qRT-PCR. A: pie chart illustrating the percent distribution of altered genes by type. B: Venn diagram demonstrating the numbers of genes altered relative to gene expression in Sham-operated rats in the 3 day Fistula and Fistula + Estrogen groups. Only a relatively small percentage of these genes were comparably changed in both groups.
Estrogen Genomic Modulation of Heart Failure

There were no significant differences in LV, RV, or lung weights in the Fistula only group compared with the Fistula + Estrogen group. However, there was a significant decrease in body weight in the Fistula + Estrogen rats relative to the Fistula group, consistent with the known effects of estrogen on metabolism.

**Genes Identified by PCR Array at 3 Days Postfistula**

Analysis of the PCR array identified a total of 55 genes with a $\geq 2.0$-fold change occurring in the Fistula group following 3 days of volume overload (Table 2). In decreasing order, the percentage of changes in gene expression in response to the imposition of the volume overload were among metabolism, receptors, membrane scaffolding and cell adhesion molecules, inflammatory/stress response, signal transduction, sympathetic nervous system, proteases, and ion channels. The relative distribution of the genes across these categories is depicted in Fig. 1A. In comparison, only 21 genes were found to be differentially modulated by estrogen treatment (Table 2, Fig. 1B). Thirteen of the genes with a $\geq 2.0$-fold change were common to both the Fistula and Fistula + Estrogen groups (Fig. 1B). This is also reflected in the heat map demonstrating the difference in expression of genes between the Fistula and Fistula + Estrogen groups (Fig. 2). Within the respective groups, there was a strong correlation between the PCR Array and qRT-PCR ($r^2 = 0.94$ and $r^2 = 0.98$, in the Fistula and Fistula + Estrogen groups; Fig. 3, A and B, respectively).

Consistent with our hypothesis, many of the genes that were upregulated in the Fistula group (i.e., $\geq 2.0$-fold increase) were normalized by estrogen treatment (i.e., $<2.0$-fold decrease). For example, genes responsible for inflammatory pathways, cytokine receptors, signal transduction, and arachidonic acid metabolism were significantly upregulated in the untreated Fistula group; however, levels of expression for these genes were statistically similar in the Fistula + Estrogen and Sham groups (Table 2, as indicated by the $<2.0$-fold change in the estrogen-treated column). Notably, the PCR array identified prostaglandin-endoperoxide synthases 1 and 2, otherwise known as COX-1 and COX-2, to be significantly upregulated in the Fistula group (7.5- and 5.3-fold, respectively; $P < 0.0001$), whereas estrogen treatment prevented the increase. TNF-α gene expression was also increased 4.2 fold for the Fistula group, but only 1.8-fold in the Fistula + Estrogen group ($P < 0.0001$). TNF-α receptor I was also significantly upregulated in the Fistula group compared with the Fistula + Estrogen group (9.2 vs. 1.2-fold, respectively; $P = 0.005$). Conversely, TNF-α receptor II gene expression was unchanged in the Fistula group (−1.4-fold) but was significantly upregulated in the Fistula + Estrogen group (8.9-fold, $P < 0.0001$). Similarly, estrogen treatment caused a marked upregulation in the expression of β1-integrin adhesion molecule, whereas there was minimal change in the Fistula group (8.9- vs. 1.6-fold, respectively; $P < 0.0001$).

**Effects of Estrogen on mRNA and Protein Levels of LV TNF-α and Receptor Subtypes at 3 Days Postfistula**

Consistent with the findings of the gene array, the untreated Fistula group had a significant increase in TNF-α mRNA expression (Fig. 4A, 2.89-fold increase compared with Sham, $P < 0.001$). In contrast, estrogen treatment prevented the increase in TNF-α mRNA. Myocardial TNF-α protein levels presented a similar trend, with significantly increased TNF-α levels in the Fistula (Fig. 4B, 8.0-fold increase compared with Sham, $P < 0.001$) prevented by estrogen treatment (Fistula vs. Fistula + Estrogen, $P < 0.001$). The findings herein are also in agreement with our previous observations that levels of TNF-α in males are increased in the initial stage of chronic volume overload (26, 31, 32). The levels of mRNA were significantly increased for TNF-α receptor I in the Fistula group, while TNF-α receptor II was significantly decreased in the LV of the untreated Fistula relative to Fistula + Estrogen group (Fig. 5, A and B, respectively; $P < 0.001$). Estrogen treat-

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Fig. 1. Heat map depicting modulation of inflammatory genes by estrogen treatment following 3 days of chronic volume overload. Shown within the brackets are the 55 top-scoring genes grouped by pattern of expression observed in Sham-operated Control, Fistula, and Fistula + Estrogen (Fistula + E2) groups. Genes in the top group have the highest magnitude of expression in Sham-operated Controls. The next group of genes had the highest expression in Fistula + E2-treated rats. The last group shows genes with maximal expression in the untreated Fistula group. The heat map is organized with group average data arranged along the x-axis, with relative ratios of expression indicated by color. Color intensity is scaled within each row, so that the highest magnitude of expression corresponds to bright red and minimal expression corresponds to bright green. The color bar at left denotes calibrated levels of expression.
Discussion

The heart’s response to chronic volume overload in male rats is a dynamic process that can be divided into three distinct phases: 1) acute myocardial remodeling, 2) compensated hypertrophic phase, and 3) decompensated heart failure (22). The acute phase consists of a marked degradation of LV interstitial collagen, thus resulting in ventricular dilatation (6). After ~2 wk of chronic volume overload, the heart transitions to an extended period of compensatory remodeling during which collagen concentration returns to normal and extensive LV hypertrophy develops (5). Once the compensatory hypertrophic mechanisms are exhausted, the transition to heart failure occurs. This is characterized by marked LV dilatation, cardiomyocyte elongation (i.e., in series sarcomeric addition), increased LV chamber compliance, and a significant reduction in systolic function (9, 11). In contrast, female rats subjected to chronic volume overload develop an essentially concentric hypertrophy without the adverse features of myocardial remodeling seen in males, affording them cardioprotection from the subsequent development of heart failure (16–19). This cardioprotection is lost in ovariectomized rats but can be largely restored by supplementary estrogen (8, 16, 18). Likewise, the adverse remodeling in male rats can be significantly attenuated by estrogen supplementation (19). However, the specific mechanisms underlying estrogen’s protective effects on the heart have thus far not been elucidated.

Given the preventative and protective effects conferred by estrogen, and since it is the initial events occurring during the acute phase of remodeling-induced postfistula in males that set in motion the long-term consequences, the time point of 3 days postfistula was used in the current study. This time point identified the inflammatory genes induced in males, affording them cardioprotection from the subsequent development of heart failure (40). As can be seen in Fig. 7A, there was no change in β1-integrin gene expression in the LV of the untreated Fistula group. Conversely, treatment with estrogen in conjunction with volume overload produced a striking upregulation of β1-integrin mRNA expression (P < 0.001). Estrogen treatment also produced a significant increase (P < 0.001) in myocardial β1-integrin at the protein level (Fig. 7B), whereas there was a reduction in β1-integrin levels in the untreated Fistula group (P < 0.001).

Estrogen Modulation of COX mRNA, Protein, and Metabolite Levels at 3 Days Postfistula

COX enzymes serve as the initial step in the prostanooid pathway (27), and changes in arachidonic acid metabolism likely influence myocardial remodeling. The mRNA levels of COX-1 and COX-2 were significantly increased in the Fistula group compared with the Fistula + Estrogen group (Fig. 6A). Consistent with this supposition, protein levels of COX-1 and COX-2 were significantly increased in the Fistula group relative to the Sham group (Fig. 6B; P < 0.001 and P < 0.05, respectively). Estrogen treatment prevented these increases at both the mRNA and protein levels for COX-1 and COX-2. The levels of PGE2 and 15d-PGJ2 were significantly elevated in the LV of the Fistula group compared with the Sham and Fistula + Estrogen groups (Fig. 6, C and D; P < 0.001 and P < 0.05, respectively).

Effects of Estrogen on β1-Integrin at 3 Days Postfistula

The cellular adhesion protein, β1-integrin, is responsible for cellular attachment to the interstitial extracellular matrix in the heart (40). As can be seen in Fig. 7A, there was no change in β1-integrin gene expression in the LV of the untreated Fistula group. Conversely, treatment with estrogen in conjunction with volume overload produced a striking upregulation of β1-integrin mRNA expression (P < 0.001). Estrogen treatment also produced a significant increase (P < 0.001) in myocardial β1-integrin at the protein level (Fig. 7B), whereas there was a reduction in β1-integrin levels in the untreated Fistula group (P < 0.001).
As can be seen in Fig. 2, of the remaining genes that were changed in response to chronic volume overload, estrogen prevented the induction of gene expression for the majority of these inflammatory genes. Focusing on those genes that were subsequently validated by qRT-PCR, the array identified significant increases in COX-1 and COX-2, TNF-α, and TNF-α receptor I gene expression postfistula. Estrogen treatment prevented or significantly attenuated these increases. Conversely, TNF-α receptor II and β1-integrin adhesion molecule gene expression was significantly upregulated by estrogen treatment.

The effects of arachidonic acid metabolism by cyclooxygenases in heart failure and tissue remodeling have been somewhat controversial with dimorphic effects on remodeling (27). Based on the observations herein, this may very well be due to underlying hormonal modulation by estrogen. Phospholipases liberate arachidonic acid from the cell membrane by hydrolyzing glycolipids (27). The results of the PCR array identified changes in 12 phospholipases at 3 days of chronic cardiac volume overload. While phospholipases have been implicated as contributing to local inflammatory responses following myocardial infarction (33), the function of these enzymes in the context of volume overload has not been established. COX-1 and COX-2 are known to be essential in the conversion of arachidonic acid to prostaglandin intermediates. These intermediates are then converted to a series of prostanooids [PGI₂, PGD₂, PGE₂, PGF₂α, thromboxane A₂ (TXA₂)] by cell-specific synthesis (15). COX-1 is constitutively expressed, while COX-2 is inducible and upregulated by proinflammatory stimuli. We found both COX-1 and COX-2 mRNA to be upregulated in the Fistula group and unchanged in the Fistula + Estrogen group. Interestingly, computer-based studies of the promoter region of COX-2 show that the classical estrogen response elements (ERE) are not present within this region (38). However, COX-2 expression is dependent on the interactions of estrogen receptors and Foxl2, a forkhead transcription factor, in the nonclassical pathway (25). COX-2 gene expression has also been shown to be an estrogen receptor (ER) and ERβ-mediated event (25, 38). We measured PGE₂ and 15d-PGJ₂ levels as an indicator of the downstream effects of estrogen on the COX enzymes and found that they were elevated in the Fistula hearts and normalized in the Fistula + Estrogen group. As the endogenous ligand for perioxisome proliferator activator receptor-gamma (14), 15d-PGJ₂ has been shown to cause increases in collagen production and proliferation in skin fibroblasts (14). However, 15d-PGJ₂ has also been shown to be antiangiogenic in bleomycin lung injury (20) and pulmonary fibroblasts (12). Other arachidonic acid metabolites such as PGI₂ have been shown to have cardioprotective effects. In mice lacking the PGI₂ receptor, they develop hypertension, LV hypertrophy, and cardiac fibrosis (13). Consistent with this potentially contributing to estrogen-mediated cardioprotection, the PCR array indicated a significant downregulation of prostaglandin I synthase mRNA levels in the Fistula group contrasted with a marked upregulation in the Fistula + Estrogen-treated hearts (−2.4 vs. 6.0-fold changes, respectively; P < 0.0001). The PCR array results also identified TXA₂ synthase and the TXA₂ receptor to be upregulated in the
Given that TXA_2_ has been shown to cause fibrosis in the heart (13), this could contribute to the rebound in collagen synthesis seen after the initial degradation in the first 2 wk postfistula (6). Thus, while there is compelling evidence suggesting that COX metabolites may be important in heart failure and that estrogen modulates key components of this pathway at the genomic level; additional studies are needed to understand their respective roles in the context of myocardial remodeling.

In the present study, we report that estrogen decreases myocardial levels of TNF-α mRNA. The TNF-α gene has an ERE located within its promoter region (1), as well as a binding site for JNK. It has been shown that estrogen can decrease TNF-α gene expression by inhibiting JNK (36). Numerous studies have implicated TNF-α in the pathogenesis of adverse myocardial remodeling in patients with end-stage heart failure (41). Evidence pointing to TNF-α being an important factor long before the development of heart failure can be construed from the observation that sustained increases in myocardial TNF-α occur within 24 h postfistula (31). Administration of pathological levels of TNF-α in normal rats produces a heart failure phenotype, with dramatic alterations in LV structure and function (3). Furthermore, we and others have shown that TNF-α inhibition using etanercept attenuates adverse myocardial remodeling in rats (2, 23). While estrogen has been shown to decrease TNF-α gene expression in heart tissue (43), a direct genomic mechanism as a possible point for regulation of myocardial remodeling by estrogen has not been substantiated prior to the results herein. However, estrogen has been shown to decrease TNF-α in myocardial tissues without myocardial stress (35) and in the ischemic brain (39).

This is also the first study linking estrogen to cardiac TNF-α receptor expression. Specifically, estrogen upregulated TNF-α receptor II and downregulated TNF-α receptor I expression at the genomic level, an effect that was also evident at the protein level. This is a critical observation as TNF-α receptors have been shown to have opposing effects on remodeling, hypertrophy, NF-κB, inflammation, and ap-
optosis in heart failure (21). In the study by Hamid et al. (21), they found that TNF-α receptor I knockout mice had less impairment of systolic and diastolic function and decreased LV remodeling postmyocardial infarction. Conversely, TNF-α receptor II knockout mice had exaggerated myocardial collagen deposition. Thus, TNF-α receptor I signaling exaggerates remodeling leading to heart failure, whereas TNF-α receptor II mediates cardioprotective effects. This sex dimorphism with respect to TNF-α receptors represents an important aspect of estrogenic modulation that warrants further study. Consistent with our observations herein, male mice overexpressing cardiac-specific TNF-α had a twofold greater increase in the TNF-α receptor I compared with the female transgenic mice (24). Accordingly, estrogen apparently regulates not only TNF-α, but also TNF-α receptors, suggesting that estrogen may switch TNF-α signaling to produce a more beneficial outcome.

Findings from our laboratory have shown that cardiomyocytes obtained from intact females postfistula maintain normal adhesion to the extracellular matrix, whereas integrin-mediated adhesion in cardiomyocytes from ovariec- tomized females is significantly reduced (10a). In the current study, we show a marked upregulation in β1-integrin mRNA and increased protein in estrogen-treated male rats subjected to chronic volume overload, in contrast to a significant reduction in the untreated Fistula group. These novel observations are the first to potentially implicate integrin-mediated adhesion as a mechanism underlying sex-related cardioprotecton. The β1-integrin subunit is responsible for cellular adhesion to extracellular matrix (e.g., collagens, laminin, and fibronectin) during myocardial development, growth, and disease states (i.e., hypertrophy) (40). Cardiomyocyte adherence to the basement membrane provides a means by which to maintain ventricular size and shape and to transduce the contractile force. Accordingly, reduced cardiomyocyte adhesion may be a mechanism contributing to wall thinning and ventricular dilatation (28, 34).

However, this is the first study to show estrogen alters integrin expression in the heart. Conversely, the β1-integrin subunit was shown to be downregulated by estrogen in angiotensin II-treated neonatal cardiac fibroblasts (37). This may be indicative of different cell type-specific responses to hormonal modulation; however, this singular finding needs to be interpreted cautiously due to the phenotypic differences between neonatal and adult fibroblasts (42). Thus, modulation of cardiomyocyte integrin expression may represent another crucial mechanism contributing to cardioprotective effects mediated by estrogen.

In summary, the findings of this study demonstrate for the first time that estrogen has the ability to normalize many of the inflammation-related genes important to cardiac remodeling that are upregulated in volume overload. Specifically, we have demonstrated estrogen-mediated genomic regulation of TNF-α, as well as divergent effects on TNF-α receptor expression, suppressing the pathological type I receptor and enhancing expression of the cardioprotective type II receptor. Furthermore, estrogen was found to genomically regulate key components of the arachidonic acid/prostanoid pathways potentially involved in adverse myocardial remodeling. Finally, the results indicate that estrogen modulates important cellular adhesion molecules at the genomic level. With changes in cardiomyocyte adhesion known to be important in myocardial remodeling, this represents another potentially key aspect of cardioprotection afforded by estrogen. Therefore, the genomic effects of estrogen may be expected to have profound effects on myocardial remodeling. However, the functions of many of these genes in the context of cardiac remodeling are largely unknown. Thus, additional studies to fully understand the impact of estrogenic regulation of these genes and their contribution to the LV remodeling response will be required.

Fig. 7. Estrogen modulation of β1-integrin adhesion molecules: fold change in mRNA levels of β1-integrin (n = 4/group) (A); and Western results of β1-integrin normalized to GAPDH (n = 6/group) (B). *P < 0.001 vs. Sham; †P < 0.001 vs. Fistula + Estrogen.
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REFERENCES


AUTHOR CONTRIBUTIONS


DISCLOSURES

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


