Impact of aging vs. estrogen loss on cardiac gene expression: estrogen replacement and inflammation

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1Cellular and Molecular Cardiology, Department of Medicine, 2Department of Medical Pharmacology, University of California, Davis; 3VA Northern California Health Care System, Mather, California; 4Department of Physiology, Second Military Medical University, Shanghai, China; 5Institute of Tropical Medicine and Infectious Diseases, University of Nairobi, Nairobi, Kenya; 6Shanghai Rongjian Biotechnology Co., Ltd., Shanghai, China; and 7Department of Physiology and Pharmacology, School of Veterinary Medicine, Texas A&M, College Station, Texas

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Pechenino AS, Lin L, Mbai FN, Lee AR, He XM, Stallone JN, Knowlton AA. Impact of aging vs. estrogen loss on cardiac gene expression: estrogen replacement and inflammation. Physiol Genomics 43: 1065–1073, 2011. First published July 12, 2011; doi:10.1152/physiolgenomics.00228.2010.—Despite an abundance of evidence to the contrary from animal studies, large clinical trials on humans have shown that estrogen administered to postmenopausal women increases the risk of cardiovascular disease. However, timing may be everything, as estrogen is often administered immediately after ovariectomy (Ovx) in animal studies, while estrogen administration in human studies occurred many years postmenopause. This study investigates the discrepancy by administering 17β-estradiol (E2) in a slow-release capsule to Norway Brown rats both immediately following Ovx and 9 wk post-Ovx (Late), and studying differences in gene expression between these two groups compared with age-matched Ovx and sham-operated animals. Two different types of microarray were used to analyze the left ventricles from these groups: an Affymetrix array (n = 3/group) and an inflammatory cytokines and receptors PCR array (n = 4/group). Key genes were analyzed by Western blotting. Ovx without replacement led to an increase in caspase 3, caspase 9, calpain 2, matrix metalloproteinase (MMP)9, and TNF-α. Caspase 6, STAT3, and CD11b increased in the Late group, while tissue inhibitor of metalloproteinase 2, MMP14, and collagen I α1 were decreased. MAD2 and fibronectin were increased in both Ovx and Late. TNF-α and inducible nitric oxide synthase (iNOS) protein levels increased with Late replacement. Many of these changes were prevented by early E2 replacement. These findings suggest that increased expression of inflammatory genes, such as TNF-α and iNOS, may be involved in some of the deleterious effects of delayed E2 administration seen in human studies.

Surgical Methods

Rats were anesthetized with 5 mg/kg ketamine and 50 mg/kg xylazine injected intraperitoneally. The rats assigned to the Sham group were anesthetized and had their body cavities opened and then immediately closed with no tissue removal; the rats assigned to the subject was 10 or more years past menopause; it has been suggested that earlier estrogen replacement might be beneficial (46).

In contrast to the human studies, several animal studies have shown that estrogen replacement is actually beneficial to the heart. Additionally, studies have shown that estrogen, given alone or in the presence of progesterone, lowers oxidative stress levels in blood vessels and tissues of young rats (49) and that estrogen is vasoprotective after vascular balloon injury in young female rats (29). Our lab has recently shown that immediate estrogen replacement in aged ovariectomized (Ovx) rats prevents deleterious changes in vascular function (40). In another study, Ovx young adult Sprague-Dawley rats (200–250 g) had increased serum levels of the inflammatory cytokines macrophage inflammatory protein (MIP1) and monocyte chemotactic protein (MCP)-1, and this was prevented by low dose 17β-estradiol (E2) (1).

The purpose of this study was to investigate changes in gene expression in aged Ovx rats with and without E2 treatment. Both immediate E2 and late E2 treatment were used to model the late E2 replacement done in clinical trials.

MATERIALS AND METHODS

Animal Model

Aged Norway Brown rats (18–22 mo old) were obtained from the National Institutes on Aging (Bethesda, MD), housed in standard female-only conditions, and fed standard laboratory rat chow. Rats were divided into four groups [Sham, Ovx, and Ovx with immediate estrogen replacement (Early), or late estrogen replacement ("Late": 9 wk post-Ovx, then 4 wk estrogen replacement)]. E2 replacement for all Early and Late was done with a subcutaneous sustained-release pellet (Innovative Research, Sarasota, FL). Uterine size and ovary absence were checked at time of tissue collection to verify treatment groups. Estrogen levels were measured by radioimmunoassay as previously described (21). All animal protocols were approved by the University of California, Davis Animal Research Committee in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Surgical Methods

The effect of estrogen loss vs. aging on cardiovascular disease is not well understood. However, there is an acceleration of atherosclerosis and a significantly increased incidence of myocardial infarction postmenopause (37). Observational studies had suggested that estrogen postmenopause was protective; however, randomized clinical trials using conjugated equine estrogen did not demonstrate a positive effect from estrogen replacement (3, 16). A key issue has been the timing of estrogen replacement (the timing hypothesis), as the average

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and the KS statistic was defined as:

$$ KS = \max_{i=1}^{n} \left( \frac{i}{n} - p_i \right), \quad p_1 \leq p_2 \leq \cdots \leq p_n. $$

The statistical significance of a GO category containing $n$ genes was evaluated by computing the empirical distribution of the LS and KS statistics in random samples of $n$ genes.

The Pathway analysis was performed using the method similar to the GO analysis, except that the genes were grouped by KEGG and BioCarta pathways, rather than GO categories (18). The pathways chosen were apoptosis, inflammation, extracellular matrix/fibrosis, vascular function/disease, signaling, and heat shock protein (Hsp) genes.

Gene Expression Chip Hybridization

The fragmented biotinylated cRNA targets were hybridized to Rat Genome 230 2.0 Array (Affymetrix, P/N 900506), which is the first whole genome array to interrogate >30,000 transcripts and variants from the rat genome, including >28,000 well-substantiated rat genes, according to the company. Two target cRNAs for each group, each containing transcripts from three rats, were hybridized to array chips. All relevant procedures including hybridization, washing, staining, scanning, and data compilation regarding the hybridization signal strength were done at the UC Davis School of Medicine Microarray Core Facility. Hybridizations were repeated once to generate two sets of data.

Real-time RT-PCR

Two-step real-time RT-PCR was performed to verify the expression of the genes listed in Table 1. Total RNA extracted from the left ventricle was reverse-transcribed into cDNA using RT reagents (Applied Biosystems, 4310179) and processed by real-time quantitative PCR with SYBR Green as double-strand DNA binding dye (Applied Biosystems, 4309155). PCR reactions were carried out in a 7900HT Sequence Detection System (Applied Biosystems), and the standard curve method was used to calculate relative expression levels. The PCR primers are listed in Table 1. They were either purchased from SuperArray Bioscience or introduced from published references and RTPrimer database (http://medgen.ugent.be/rtprimerdb/index.php). β-Actin was employed as an internal control (Microarray showed no change in β-actin). Results were expressed as ratios relative to the amount of β-actin.

PCR Array

To further investigate changes in inflammatory genes that might have been missed by the Affy array, the Inflammatory Cytokines and Receptors PCR array (SABiosciences) was used, along with the cDNA synthesis kit and the SYBR green double-stranded DNA detection kit. This PCR-based array is performed precisely like real-time PCR, except that the primers are preloaded into the PCR plate wells. The PCR was performed on an ABI Prism Sequence Detection System (Applied Biosystems), and the data were analyzed using the RT2 Profiler PCR Array Data Analysis (SABiosciences, available online at http://www.sabiosciences.com). Gene expression levels were normalized to ribosomal protein, large, P1 (Rplp1) expression levels, which did not change between groups and are reported as fold of sham. Only those genes whose expression levels showed a 1.5-fold or greater expression difference between groups and had a $P$ value of <0.20 were considered significant changes between groups. To select candidate gene changes, a $P$ value of 0.20 was chosen due to the variation on the arrays resulting from a low sample size number (25, 29). Since only three arrays were performed for each group, it was necessary to increase the $P$ value over the traditional 0.05 to have a representative sampling of genes whose expression was altered on the array and eliminate the sampling errors that come from a small sample size.

Western Blot Analysis

Analysis was performed as previously described (13), except for fibronectin Westerns, where a special approach was used to solubilize this large protein, as previously described (4). Antibodies were used in the following dilutions: TNF-α, 1:500 (Santa Cruz Biotechnology,
no. 5 (Early group: Ovx followed by immediate estrogen replacement), and column 6 (Late group: Ovx followed by delayed estrogen replacement). *PCR performed on genes not on original Affymetrix array.

Santa Cruz, CA); MIP-1β 1:1000 (PeproTech, Rocky Hill, NJ); inducible nitric oxide synthase (iNOS), 1:500 (BD Biosciences, San Jose, CA); endothelial (e)NOS, 1:500 (Cell Signaling, Danvers, MA); fibronectin, 1:5000 (Rockland Immunochemicals, Gilbertsville, PA); caspase 9, 1:2000 (Abcam, Cambridge, MA); tissue inhibitor of metalloproteasease (TIMP)2, 1:1000 (Millipore, Billerica, MA); heme oxygenase-1, 1:2000 (Cell Signalig); chemokine (C-X3-C motif) ligand 1 (CXCRL1), 1:5000 (Abcam); MCP-1, 1:5000 (Abcam); ABCF1, 1:1000 (Abcam); endothelial monoocyte activating polypeptide II (EMAP II) 1:1000 (Abcam); GAPDH, 1:5000 (Fitzgerald, Concord, MA), and the appropriate horseradish peroxidase-conjugated secondary antibodies of anti-mouse or anti-rabbit (GE Healthcare UK), were used at 1:1000 dilutions and developed using the West Pico enhanced chemiluminescence (Thermo Scientific, Waltham, MA). Proteins were normalized to GAPDH, which did not vary among groups.

Data Analysis

Changes in gene expression levels seen with real-time PCR and Western blot analyses were statistically analyzed using an ANOVA on Ranks, followed by a Student-Newman-Keuls test or Dunn’s test, where appropriate, with P < 0.05 considered significant. See Supplementary Methods for full details of Affymetrix Array analysis.1

RESULTS

Model

Norway Brown rats were chosen for this work because they are one of several strains, including Fischer rats, that are considered to be ideal aging models. The Norway Brown and the Fischer rat both age without concomitant obesity. Thus one can study aging without a key confounding factor, obesity. The timing of treatments was based on previous work, where we found that post-Ovx cardiac HSP 72 levels took 9 wk to drop to the levels found in males (48). Likely this represents a cascade of changes that occur postloss of estrogen. Therefore, we waited 9 wk post-Ovx before collecting samples. To investigate the effect of late replacement added to the gene expression defined at 9 wk post-Ovx vs. post-Ovx with immediate replacement, we added an additional group of rats with delayed

1 The online version of this article contains supplemental material.
estrogen replacement group for 4 wk. Tissues were collected from these rats at 13 wk. The results of the Women’s Health Initiative (WHI) and other studies suggest that late replacement of estrogen is deleterious. To further understand this distinction, we incorporated both immediate estrogen replacement (Early) and delayed estrogen replacement (Late) into our study design. The serum E2 was measured to verify treatment groups (Fig. 1) and showed no statistically significant difference between the E2 levels in the Early and Late replacement groups, but the expected significant decrease in serum E2 levels upon Ovx.

**Affymetrix Array and Apoptosis Pathway**

Genes changing in the left ventricle will reflect not only changes in cardiac myocytes but also changes in the coronary arterial cells, in fibroblasts, and in leukocytes and other cell types that may be present in the heart at time of tissue collection. Using an Affymetrix array containing 10,000 genes, we found a number of genes whose expression levels differed among the groups twofold or more. As discussed in MATERIALS AND METHODS, a subset of KEGG/BioCarta pathways was chosen for further analysis by real-time PCR. These included apoptosis, inflammation, extracellular matrix/fibrosis, and vascular function/disease genes. For the apoptosis pathway, a number of changes in apoptosis genes were identified and confirmed by real-time PCR (Table 1). Apoptosis-associated factor (APAF1) was greater in Sham and Ovx groups than the two E2 replacement groups. Caspase 3 was nearly tripled in Ovx vs. all (P < 0.05). Similarly, caspase 9 and calpain 2 were increased in the Ovx group (P < 0.05). MADD was highest in the Late group and in both Ovx and Late was significantly higher than sham (P < 0.05).

**Inflammation Pathway**

IL-6R1 and TNF-α were increased in Ovx (P < 0.05). iNOS was not detected by the Affymetrix array, but given its importance in cardiovascular disease, its expression was examined by real-time PCR. iNOS was found to be significantly increased in Ovx (Table 1, P < 0.05). MIP-1 and STAT3 were increased in the Ovx group on the array but, by real-time PCR, were significantly higher in the Late group (Table 1, P < 0.05). SOCS2, SOCS3, and soluble epoxide hydrolase were investigated as we have previously observed these genes to change in young rats, but there were no differences for any of these genes (14).

**Extracellular Matrix Pathway**

For genes involved in the extracellular matrix pathway, connexin 43 and MMP9 were decreased in the Ovx group (Table 1, P < 0.05). Collagen1 α1 was decreased in the Late group. Fibronectin was higher in both the Ovx and Late group, while fibromodulin was unchanged (Table 1). Both MMP14 and TIMP2 were decreased in the Late group (P < 0.05).

**Vascular Function and Disease Pathway**

Examination of the vascular function/disease pathway showed that ACE tended to be higher in the Late group, but this did not reach significance. Integrin-αM (also known as CD11b) was increased in the Late group (Table 1, P < 0.05). Soluble guanylyl cyclase (sGC) -α and -β were decreased with Ovx, in contrast to our findings in the young adult rat, where these did not differ from sham (14).

Although we found a number of changes with the rat Affymetrix array, we were concerned because there were a number of gene sequences for which the nomenclature exists as Caenorhabditis elegans, Drosophila, or other organisms that may show very little similarity to the rat gene. In addition, the rat array contained quite a few estimated sequence tags (ESTs), for which the gene they encode is as yet uncharacterized. For example, the Affymetrix array contains six sequences that code for fibronectin, but only two have been confirmed for fibronectin, while the other four are ESTs that are considered weakly or moderately similar to fibronectin. We were concerned that given this “noise” we might miss important changes. Therefore, it was decided to use an additional approach to investigate changes in proinflammatory genes.

**Inflammatory Gene PCR Array**

Inflammation is a major issue in aging and estrogen loss. As such, we chose to perform a more in-depth analysis of the inflammatory pathway using the SABiosciences Inflammatory Cytokines and Receptors PCR array. This array system has the advantage that there are only 89 genes total and all of the sequences are derived from the rat sequences. There are no ESTs, and every primer set corresponds to a verified rat gene. Software supplied by the company allowed analysis of each group compared with the rest.

Late E2 replacement affected the expression of a limited number of genes on the PCR array (Table 2 and Supplementary Table S2). MCP-1 expression by the PCR array was 49-fold lower vs. Sham, while MIP-1β was 3.9-fold higher vs. Ovx (Table 2), similar to findings on the Affymetrix array. EMAP II, also known as Scye 1, was increased 84-fold in the Late group compared with sham and slightly less in the Ovx group.

Surprisingly, the expression levels of several inflammatory genes in the Ovx group were much lower than those in the Sham group (Table 3 and Supplementary Table S3). Cx3cr1, also known as fractaline, which promotes leukocyte transmigration into the arterial wall and thus atherosclerosis (24), was decreased 13.6-fold vs. the Sham group and 2.1-fold vs. the Early group. Cx3cr1 was decreased even more in the Early group vs. sham (29-fold). Thus, Ovx decreased expression of Cx3cr1 regardless of immediate E2 replacement. MIP-1β, a proinflammatory gene (50), was less in the Ovx group than either the Early or Late group. Lastly, MCP-1, a monocyte adhesion protein (52), was 51.5-fold lower in Ovx than Sham.
Table 2. PCR array: all groups vs. Late

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sham</th>
<th>Ovx</th>
<th>Early</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mcp1</td>
<td>49.86</td>
<td>−3.97</td>
<td>1.22</td>
</tr>
<tr>
<td>Mip1β</td>
<td>−84.12</td>
<td>1.22</td>
<td></td>
</tr>
<tr>
<td>Emap II</td>
<td>−3.87</td>
<td>1.22</td>
<td></td>
</tr>
</tbody>
</table>

PCR array compared with Late. *Column 1*, gene name; *column 2*, fold-change of Sham compared with Late; *column 3*, fold-change of Ovx compared with Late; *column 4*, fold-change of Early compared with Ovx. Numbers are significantly different (P < 0.2) as compared with Late. The complete list of genes with altered expression is in Supplementary Table S2.

A key question in our study was whether immediate E2 replacement could prevent deleterious changes seen with Ovx. Genes on the PCR array with altered expression by immediate E2 replacement (Early) are listed in Table 4 and Supplementary Table S3. MIP-1β was 2.7-fold higher in the Early vs. Ovx, but 3.9-fold lower than the Late group (Table 4). Cx3cr1 levels were 29-fold less in the Early vs. the Sham and 2.1-fold less in the Early vs. the Ovx (Table 4). Similarly, MCP-1 levels were decreased 17-fold vs. Sham.

**Inflammatory Gene Corresponding Protein Changes**

Western blotting was done for selective key inflammatory proteins to determine if the protein changed as well as the mRNA. TNF-α levels were doubled in the Late group, while there was no change in the Ovx group vs. Sham (P < 0.05, Fig. 2A). iNOS, which was not detected on the Affymetrix array, but found to be increased in the Ovx group by real-time PCR (Table 1), was also increased at the protein level, but only in the Late group (Fig. 2B). Although MIP-1β was less in the Ovx group than either the Early and Late group on the PCR array (Table 3), MIP-1β protein levels tended to be higher in Ovx and Early (Fig. 2C), but this was not significant. mRNA for MCP-1 was 51.5-fold lower (Table 3) in Ovx and 17-fold lower in Early (Table 4) compared with Sham; however, by Western blot only the Ovx MCP-1 protein levels were decreased significantly (P < 0.05) compared with Sham (Fig. 2D). Fibronectin, a key extracellular matrix protein that is thought to be proinflammatory, was increased in Late and Ovx groups by real-time PCR, but Western showed no significant difference in fibronectin expression among the groups (Fig. 3A). Cx3cr1 was decreased in Ovx and in the Early groups compared with Sham by PCR array (Table 3), but the protein levels were unchanged (Fig. 3B). Emap II, a proapoptotic cytokine, was significantly decreased in the Late group by Western (Fig. 3C), a difference from the increase in mRNA seen with this group. The Affymetrix array also showed increases in caspase 6, STAT3, and CD11b in the Late group.

Additional real-time PCR studies demonstrated that iNOS was increased at the mRNA level in the Ovx group, but at the protein level, iNOS was markedly increased in the Late group only. In contrast TIMP2, MMP14, and collagen 1 α1 were decreased in the Late group, which may have an adverse effect on ventricular remodeling. Ovx without replacement led to an increase in caspase 9, calpain 2, MMP9, and TNF-α. MADD and fibronectin were increased in both Ovx and Late group, but this did not translate into a change in fibronectin protein levels. Many of these changes were prevented by early E2 replacement.

**DISCUSSION**

The left ventricle, which was the focus of the current study, includes not only cardiac myocytes, which constitute most of cardiac mass, but also endothelial cells, smooth muscle cells, fibroblasts and leukocytes. Late replacement of estrogen was characterized by increased expression of CD11b, MIP-1β, STAT3, EMAP II, fibronectin, caspase 6, and MADD. TNF-α and iNOS protein levels increased with Late replacement even though the RNA levels were not increased for these groups. These changes involve predominantly proinflammatory and proatherosclerotic proteins. TNF-α’s proinflammatory effects include upregulation of the adhesion molecules ICAM-1, VCAM-1, and E-selectin in endothelial cells, enhancing the recruitment of leukocytes (11, 25, 45). TNF-α also increases oxidative stress by activation of NADPH oxidase (9). iNOS, another proinflammatory gene, was increased markedly at the protein level only in the Late group. CD11b has a central role in leukocyte adhesion and atherosclerosis (37). MIP-1β promotes leukocyte adhesion and atherosclerosis (43) and has been found to have a negative role in postinfarction remodeling...
Table 4. PCR Array: all groups vs. Early

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sham</th>
<th>Ovx</th>
<th>Late</th>
<th>Fold Change</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mcpi</td>
<td>16.97</td>
<td>-2.72</td>
<td>3.87</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cx3cl1</td>
<td>29.00</td>
<td>2.13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Emap II</td>
<td>-35.51</td>
<td></td>
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</tbody>
</table>

PCR array compared with Early. Column 1, gene name; column 2, fold-change of Sham compared with Early; column 3, fold-change of Ovx compared with Early; column 4, fold-change of Late compared with Early. Numbers are significantly different ($P < 0.05$) as compared with Early. The complete list of genes with altered expression is in Supplementary Table S4.

(22). STAT3 has a complex role in the heart and can be either protective or proinflammatory (15). Fibronectin, an extracellular matrix protein found in the plasma, is expressed in atherosclerotic plaques and in the fibrous cap (5). Fibronectin is considered to have proinflammatory properties but also has key functions in wound repair and scar formation (11, 20). Additionally, increased fibrosis in the heart, caused in part by an increase in the expression of fibronectin, leads to left ventricular stiffness and diastolic dysfunction, a predominant cause of heart attacks in the aging population (19). Recently it has been reported that estrogen blocks cardiac fibrosis via estrogen receptor-β, inhibiting the increase in fibronectin, vimentin, and collagen that occurs with angiotensin II (34). EMAP II is considered a proinflammatory cytokine (44), as discussed below, while MADD and caspase 6 promote apoptosis. Thus, these changes in gene expression with late E2 replacement help to explain why in human studies E2 replacement many years postmenopause was associated with an increase in cardiovascular events.

**TNF-α**

TNF-α is a potent proinflammatory cytokine whose circulating levels increase with age and heart disease. Our lab has previously shown that TNF-α RNA levels increase with Ovx in young adult rats (14). Suzuki et al. (42) reported that after Ovx cerebral levels of TNF-α after middle cerebral artery occlusion (MCAO) were lower in mice with immediate E2 replacement. There was no difference in cerebral TNF-α levels after MCAO in mice with late E2 replacement vs. no replacement; however, basal TNF-α levels were not measured (42). In postmenopausal women, estrogen treatment inhibited the release of TNF-α from monocytes (31). Primary human bone marrow cultures collected within 5 yr postmenopause showed no change in TNF-α expression levels, whereas those from women recently discontinuing hormone replacement had elevated TNF-α (36). Thus, overall estrogen replacement decreases TNF-α levels and estrogen loss increases TNF-α levels, and the current study supports a marked increase in TNF-α with late estrogen replacement.

iNOS

iNOS expression was markedly increased at the protein level only in the Late replacement group. Increased iNOS occurs with aging in general; however, among the four aged groups, iNOS protein levels were clearly increased only in the Late group (10). iNOS is proinflammatory and can lead to an overproduction of NO, which then combines with superoxide ion ($O_2^-$) to generate peroxynitrite. Peroxynitrite is a potent oxidizing and nitrating agent that has been shown to contribute to myocardial and vascular dysfunction in many diseases (30). Peroxynitrite has been implicated in the inactivation of a plethora of enzymes, including antioxidants. Increased NO can also lead to protein s-nitrosylation (47). S-nitrosylation may result in protein dysfunction but at times can also be protective, as recently reviewed (12). Lastly, increased iNOS also leads to the production of inflammatory cytokines and apoptosis.

**Ovx and Cardiac Gene Changes**

Ovx without estrogen replacement was associated with increased expression of caspase 3, caspase 9, calpain 2, MADD, IL-6R1, iNOS, and fibronectin. These genes are primarily proapoptotic, proinflammatory, and profibrotic. MCP-1 is one of the circulating chemokines that recruits monocytes, macrophages, and T-cells to areas of inflammatory injury (8). Similar to other chemokines discussed below, MCP-1 is found in areas of atherosclerotic lesions and contributes to the recruitment of inflammatory cells into the arterial wall (26). In the current study, MCP-1 surprisingly decreased in all groups compared with sham by PCR array, but Western showed a decrease only in the Early group ($P < 0.05$). Others have shown that in 19 wk old mice MCP-1 protein levels decreased in areas of cerebral ischemia with immediate E2 replacement, but delayed E2 treatment did not alter the protein levels (6), a finding consistent with the current study.

**Soluble Guanylyl Cyclase and Aging**

sGC, both the α- and β-subunits, decreased by both the Affymetrix array and real-time PCR only with Ovx. This is consistent with our previous finding that sGC mRNA and protein decreased in the aorta only with the combination of aging and Ovx, and this could be prevented by E2 replacement (40). Loss of sGC results in impaired vascular relaxation, which is critical for normal vascular function. In contrast, in the young adult Sprague-Dawley rat, sGC was unchanged post-Ovx (14). Early E2 replacement prevented many of the changes associated with Ovx, including the down regulation of sGC-α and -β.

**Cx3cl1 and Inflammatory Signaling**

Cx3cl1 is an inflammatory signaling receptor important in monocyte recruitment. Cx3cl1-mediated signaling leads to increased expression of ICAM-1 and neutrophil adhesion on endothelial cells (50). Cx3cl1 was decreased in both the Ovx...
and Early groups vs. sham, but not in the Late group. Thus, Ovx decreased expression of Cx3cr1 independently of E2 replacement. Late E2 reversed this change.

**EMAP II**

EMAP II is a proinflammatory cytokine that is released in response to stress. In the vasculature, EMAP II is increased after percutaneous coronary intervention (PCI), and EMAP II enhances the recruitment of inflammatory cells (44). Treatment with rapamycin prevents neointima formation and the increase in EMAP II expression post-PCI (27). EMAP II also inhibits angiogenesis, which can be beneficial in tumors but can be deleterious when manifest as inhibition of endothelial cell proliferation. EMAP II binds to VEGF receptor 1 and 2,
blocking downstream signaling from VEGF (6). The effect of estrogen on EMAP II has not been studied. Our data indicate that treatment with E2 Early or Late after Ovx causes an increase in EMAP II mRNA, but the protein levels of EMAP II are decreased with Late E2 treatment and unchanged with Early E2 replacement.

**Affymetrix Array vs. PCR Array**

There was a disparity between the Affymetrix Array, real-time PCR done for the Affymetrix Array and the PCR Array. Both the Affymetrix Array and real-time PCR done at that time showed an increase in TNF-α mRNA. Previously we have found that TNF-α increased in young adult Sprague-Dawley rats 9 wk post-Ovx (14). In contrast to the Affymetrix array and real-time PCR where an increase in TNF-α was seen with Ovx, TNF-α RNA levels were unchanged on the PCR array (not shown). For our first real-time PCR we used primers selected from RTPRimerDB (http://medgen.ugent.be/rtprimerdb/index.php), a database of confirmed primers including specific primers for the Norway Brown rat. The PCR array uses proprietary primers, so the sequence is unknown. Differences in primers may account for the differences.

**mRNA vs. Protein**

There was a divergence between RNA findings and protein expression, which is not unexpected given the complexity of regulation of gene expression and translation. Only for MCP-1 do changes in RNA levels coincide with the protein levels. This lack of correlation between mRNA and protein is not surprising as real-time PCR is exquisitely sensitive compared with Western blotting, where it is challenging to convincingly show a 40% change. Also, many of the changes were seen in cytokines, which often have short half-lives as proteins. Finally, the turnover of both RNA and proteins is differentially regulated. Gene expression is controlled at multiple levels including transcription, posttranscription stability, translation, and posttranslational degradation. These conditions likely contributed to the differences between mRNA and protein expression level findings, as PCR is definitively more sensitive than protein analysis by Western blot.

This is the first study to our knowledge that investigates the effect of late estrogen replacement in aged rats post-Ovx as a model of menopause. A significant number of gene changes were found, many of these characterized as proinflammatory. A consistent finding was changes in genes that promote leukocyte attraction and adhesion, early steps in atherosclerosis. The most important finding is the increase in proinflammatory and proapoptotic proteins with late estrogen replacement, exemplified by the increase in TNF-α and iNOS protein levels with late replacement. As TNF-α increases the expression of adhesion molecules along with other proinflammatory effects, these findings suggest a mechanism for the increase in cardiovascular disease seen after delayed conjugated equine estrogen replacement in clinical trials such as the WHI. Further work will be needed to fully understand the effects of estrogen and its absence on the aging heart.

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**DISCLOSURES**

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

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