Estrogen Modulates Inflammatory Mediator Expression and Neutrophil Chemotaxis in Injured Arteries

Andrew P. Miller, MD; Wenguang Feng, MD, PhD; Dongqi Xing, MD, PhD; Nathaniel M. Weathington, BS; J. Edwin Blalock, PhD; Yiu-Fai Chen, PhD; Suzanne Oparil, MD

Background—We have previously shown that estrogen (17β-estradiol; E2) inhibits neointima formation and migration of leukocytes, particularly neutrophils, into rat carotid arteries after acute endoluminal injury. This study tested the hypothesis that E2 inhibits expression of adhesion molecules, chemokines, and proinflammatory cytokines in rat carotid arteries in the early hours after balloon injury, thus attenuating the stimulus for leukocyte entry and negatively modulating the injury response.

Methods and Results—Ovariectomized (OVX) rats were randomly assigned to treatment with E2 or vehicle (V) and subjected to balloon injury of the right carotid artery. After 2, 6, and 24 hours, rats were euthanized, and both carotid arteries were processed for real-time reverse transcription–polymerase chain reaction (2 and 24 hours), ELISA (6 hours), or neutrophil chemotaxis assay (24 hours). Expression of mRNA for adhesion molecules (P-selectin, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1), chemokine-1 (CINC)-2β and monocyte chemoattractant protein (MCP)-1, and proinflammatory cytokines (interleukin [IL]-1 and IL-6) was markedly increased (2 to 5000 times) in injured arteries of OVX + V rats at 2 hours and was reduced by 24 hours. E2 significantly attenuated expression of the proinflammatory mediators (by 60% to 80%) at 2 hours. ELISA confirmed injury-induced upregulation of neutrophil and monocyte/macrophage chemokine (CINC-2α, MCP-1) and distinctive expression of CINC-2α in OVX + V arteries and E2-induced inhibition of CINC-2α expression. E2 significantly (by 65%) inhibited neutrophil chemotactic activity of arterial homogenates.

Conclusions—E2 attenuates the early vascular injury response, at least in part, by negatively modulating proinflammatory mediator expression and the resultant chemotactic activity of injured vessels for neutrophils. (Circulation. 2004;110:1664-1669.)

Key Words: restenosis ■ inflammation ■ hormones ■ vessels ■ women

Inflammation has long been implicated in the pathogenesis of many forms of vascular disease. Furthermore, there is growing evidence that recruitment and activation of neutrophils (granulocytes) within the coronary circulation participate in myocardial injury in acute coronary syndromes and that adhesion and tethering of neutrophils to damaged endothelium are involved in the earliest stages of atherogenesis.

In animal models of acute vascular injury, inflammation contributes to remodeling and restenosis. In a porcine coronary artery stent restenosis model, the extent of the inflammatory response correlated strongly with the degree of vascular injury and the development of neointima. Furthermore, balloon overstretch injury of porcine coronary arteries has been shown to elicit accumulation of neutrophils in the adventitia surrounding the injury site as early as 0.5 hour after injury. In that study, infiltration of the adventitia by neutrophils peaked at 6 hours after injury and then declined, to be followed by a wave of macrophages that peaked at 3 days.

Expression of mRNAs for cell adhesion molecules, including P-selectin, E-selectin, and vascular cell adhesion molecule-1 (VCAM-1), and of the neutrophil-specific chemotactic cytokines alveolar macrophage chemotactic factor-1/cytokine-induced neutrophil chemoattractant (AMCF-1/CINC) and AMCF-2, are also observed in adventitial and perivascular tissues.

Our laboratory has utilized balloon injury of the rat carotid artery to study vascular injury responses and their modulation by ovarian hormones in vivo. We have demonstrated entry of large numbers of granulocytes (neutrophils) and macrophages/macrophages from periadventitial tissues into the adventitial domain of arteries within the first 24 hours after injury in ovariectomized (OVX) rats. The granulocyte and monocyte/macrophage populations of injured arteries, assessed by flow cytometry, decline markedly by 3 days after injury and remain low at 7 days. Treatment with estrogen (17β-estradiol; E2) markedly inhibits the adventitial and

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perivascular infiltration of leukocytes (predominantly granulocytes and monocytes/macrophages) in injured arteries at the 24-hour time point. The E2 effect on leukocyte subtypes in injured arteries is not apparent at the 3- and 7-day time points. These findings are consistent with our previous observations that E2 negatively modulates neutointima formation in balloon-injured rat carotid arteries by mechanisms that are fully expressed in the early period (first 3 days) after the insult.13

The present study elucidated the E2-inhibitable mechanisms by which neutrophils and monocytes/macrophages are recruited to the adventitial domains of arteries after endothelial injury. Specifically, we tested the hypothesis that adhesion molecules, proinflammatory cytokines, and chemokines that are selective for neutrophils and monocytes/macrophages are expressed at high levels in balloon-injured carotid arteries of OVX rats in the early hours after the insult; that extracts of these arteries are chemoattractant for neutrophils; and that E2 treatment inhibits expression of these chemical mediators in injured arteries as well as the chemotactic activity of arterial extracts.

Methods

Animal Preparation
Female Sprague-Dawley rats were obtained from Charles River Breeding Laboratories (Wilmington, Mass) at 10 weeks of age; were maintained at constant humidity (60±5%), temperature (24±1°C), and light cycle (6 AM to 6 PM) conditions; and were fed a standard rat pellet diet (Ralston Purina Diet) ad libitum. All protocols were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham and were consistent with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). All rats were subjected to OVX and randomly assigned to an overdose of sodium pentobarbital (75 mg/kg) at 2 and 24 hours after injury; both carotid arteries were rapidly dissected and removed; and total RNA was extracted for analysis by real-time reverse transcription-polymerase chain reaction (RT-PCR). In subsequent studies, OVX +V and OVX+E2 rats were humanly killed at 6 hours after injury (n=8 per group), and carotid arteries were removed and processed for protein measurements, or at 24 hours (n=3 per group) for bioassay of neutrophil chemotactic activity. No attempt was made to remove the adventitia or perivascular connective tissue from any artery.

Real-Time, Quantitative RT-PCR Analysis of Inflammatory Mediators
RNA was extracted from 20 mg of tissue (1 to 3 vessels pooled per sample) with TRIzol (Invitrogen) treated with DNAase I to remove genomic DNA and then purified with use of an RNA purification kit (Invitrogen). The protein- and DNA-free RNA was reverse-transcribed to cDNA with use of the SYBR Green RT-PCR kit (Applied Biosystems) and specific primers (Table 1). cDNA was amplified by PCR in the iCycler for 40 cycles, and relative RNA levels were calculated with iCycler software and a standard equation (Applied Biosystems). Unknowns were normalized against ribosomal protein S9 mRNA, because expression of this housekeeping gene has been shown to remain stable in a rat carotid artery injury model14 and then standardized to the OVX+V–injured value because the relative expression of inflammatory mediator mRNA was highest in this group.

Inflammatory Mediator Protein Measurement
Samples for protein measurement were prepared from single injured right and 3 pooled left carotid arteries (n=8 per group). Arteries were homogenized with a glass homogenizer in 200 µL of lysis buffer with a protease inhibitor (Pierce). After centrifugation at 1500g for 15 minutes, supernatants were collected and protein concentration was quantified by the bicinchoninic acid assay. Protein levels of 6 chosen cytokines/chemokines were quantified by multiplexed sandwich ELISA (Searchlight rat cytokine array, Pierce) at a core laboratory. Results were normalized to protein concentration from the bicinchoninic acid assay.

Chemotaxis Assay
To assess chemotactic activity of arterial homogenates, in vitro chemotaxis assays were performed on isolated human neutrophils in a 96-well-modified Boyden chamber appropriate for the evaluation of leukocyte chemotaxis. Human neutrophils were isolated from peripheral blood by standard methods with Histopaque 1077 and 1119.13 Cells were washed twice with Hanks’ balanced salt solution with 1% bovine serum albumin, counted, and resuspended at 2×10⁶ cells/mL in Dulbecco’s modified Eagle’s medium with 5% bovine serum albumin (all chemicals from Sigma except where noted). In vitro assays were then performed in a 96-well polycarbonate filter plate with a 3-µm pore size appropriate for leukocyte chemotaxis.

Table 1: Specific Sense and Antisense Primers for Quantitative, Real-Time RT-PCR Analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sense Sequence</th>
<th>Primer Antisense Sequence</th>
<th>PCR Product, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>CINC-2β</td>
<td>TCAGGCACTGTGTGG</td>
<td>TGACTTCTGTCTGGTG</td>
<td>127</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>CAAGGGGAGATGAGG</td>
<td>TGGCGGTAAATAGTTGATAT</td>
<td>183</td>
</tr>
<tr>
<td>IL-10</td>
<td>AAAGCAGGCAGTCAGCAGC</td>
<td>TCAACACTCATCTGGCTCTTG</td>
<td>81</td>
</tr>
<tr>
<td>IL-1β</td>
<td>GGAATAGACGCAGACTCGC</td>
<td>CTTGTTTGGCTATTTTTCTG</td>
<td>146</td>
</tr>
<tr>
<td>IL-6</td>
<td>CAAAGCAGGTCATGAGAGG</td>
<td>GGTCTTCTGAGGCTTCTTGG</td>
<td>151</td>
</tr>
<tr>
<td>MCP-1</td>
<td>ATGCAGGTCTCTGTCACGCT</td>
<td>GGTGCTGAGGTCCTTAGGGT</td>
<td>345</td>
</tr>
<tr>
<td>P-selectin</td>
<td>ATGAAATCGCTCACCTC</td>
<td>TTAATGGGCTCTTGGCTT</td>
<td>152</td>
</tr>
<tr>
<td>Ribosomal protein S9</td>
<td>GCGGAGTTCCGTTGTCG</td>
<td>CAGAGGCTACAGAGGCTTCCG</td>
<td>200</td>
</tr>
<tr>
<td>TNF-α</td>
<td>CTTACTACCTCCCCAGGTTCTTCA</td>
<td>GAGACTCCCAGGTTACATGG</td>
<td>192</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>GGGGATTCGTTGTTCTT</td>
<td>CAGGGCTCAAGGTCT</td>
<td>136</td>
</tr>
</tbody>
</table>

Abbreviations are as defined in text.
Cell solution (100 μL H9262) was added to each well in the top filter plate portion of the assembly, and 150 μL of a 1:10 dilution of arterial homogenate in Dulbecco’s modified Eagle’s medium was added to the bottom feeder wells. After 1 hour the upper portion was removed, and 3 photomicrographs (×11003) per well were digitally recorded on an Olympus IX70 microscope and Perkin-Elmer Ultra-view image capture equipment. Cell counts were made from these images. For ease of comparison of results between experiments, data were standardized to a chemotactic index with cell migration to control uninjured artery as a baseline, eg, index = (mean cells per field migrating to injured artery / mean cells per field migrating to uninjured artery).

### Statistical Analysis

Results are expressed as mean±SEM. Data were evaluated by 1-way ANOVA. When the overall F test of the ANOVA was significant, a multiple-comparison Dunnett test was applied. A Student t test was used in 2-mean comparisons. Differences were reported as significant at a value of P<0.05.

### Results

Real-time quantitative RT-PCR analysis of 2-hour control and injured carotid arteries from OVX+V rats showed that all mediators were expressed at very low levels in uninjured vessels and that mRNA levels increased markedly (range, 2- to 5000-fold) after injury (Table 2). E2 treatment resulted in significant reductions of mRNA levels of all adhesion molecules examined (P-selectin, VCAM-1, intercellular adhesion molecule [ICAM]-1), as well as the cytokines interleukin (IL)-1 and IL-6, the neutrophil-selective chemoattractant CINC-2β, and the monocyte/macrophage-selective chemoattractant monocyte chemoattractant protein (MCP)-1 at 2 hours after injury (Figure 1, top). Expression of IL-10 and tumor necrosis factor (TNF)-α was not altered.

![2 Hours Post Injury](image1)

![24 Hours Post Injury](image2)

**Figure 1.** Effects of E2 (20 μg · kg⁻¹ · d⁻¹) on mRNA expression of adhesion molecules, chemokines, and cytokines in balloon-injured right common carotid arteries of OVX rats at 2 hours (top) and 24 hours (bottom) after injury compared with OVX+V rats. Data, expressed as mean±SEM, are from 4 to 8 real-time quantitative RT-PCR assays with 1 to 3 vessels (pooled) per sample for each assay and are standardized to mean mRNA level of V-treated injured samples. *P<0.05 vs respective injured carotid arteries from OVX+V rats. Abbreviations are as defined in text.
IL-6 mRNA levels (Figure 1, bottom). Interestingly, steady-state mRNA levels for the chemokine levels for CINC-2α and MCP-1 remained suppressed in injured arteries of E2-treated rats compared with V-treated controls, suggesting a sustained effect on leukocyte chemotaxis.

In a preliminary study, levels of MCP-1 protein were quantified by ELISA at 6 and 24 hours after injury. An important injury response was demonstrated, with significant increases in MCP-1 at 6 and 24 hours (100- and 20-fold, respectively). Importantly, levels of MCP-1 trended downward at 24 hours. In subsequent studies, the 6-hour time point was chosen for measurement of inflammatory mediator protein levels.

Protein levels of 6 selected chemokines and cytokines were quantified in 6-hour control and injured arteries of V- and E2-treated rats by multiplexed sandwich ELISA (Table 3). All chemokines and cytokines were detectable in uninjured vessels, and expression of all except IL-6 was significantly increased (range, 2- to 20-fold) by injury. Importantly, the neutrophil-specific chemokine CINC-2α and the monocyte/macrophage-specific chemokine MCP-1 were expressed at very low levels (26 and 560 pg/mg protein, respectively) in uninjured arteries, and expression was greatly amplified (12- and 20-fold, respectively) at 6 hours after injury. E2 treatment significantly (by 43%) reduced CINC-2α levels only.

Because both mRNA and protein levels of CINC-2 in uninjured arteries were reduced in the E2-treated groups at all time points tested, in vitro chemotactic analysis of human neutrophils to arterial homogenates was performed to assess the functional significance of the alteration in chemokine expression (Figure 2). A significant injury effect was demonstrated, with marked (2-fold) increases in neutrophil migration toward homogenates of injured vessels. Chemotactic activity in injured arteries from E2-treated animals was significantly reduced (by 65%, P<0.01). Chemotactic activity was normalized to uninjured arterial homogenate as described in Methods. Chemotactic activity of uninjured vessels from V and E2 groups was not significantly different (data not shown).

**Discussion**

The cascade of events that characterizes vascular inflammation has been well described. An initial vascular insult leads to adhesion molecule expression, with rolling and binding of leukocytes to the vessel wall, then to leukocyte infiltration guided by chemotactic factors, and finally to further recruitment of inflammatory cells via release of cytokines. The present study places this inflammation model in the context of acute vascular injury by demonstrating important injury-induced upregulation of inflammatory mediators in all 3 model phases and provides direct in vivo support for estrogentic vasoprotection mediated through an antiinflammatory pathway. Previous in vitro and in vivo studies in animal models and assays of plasma specimens from humans treated with E2 have suggested such a relation.

E2 has been shown to modulate adhesion molecule expression in response to cytokine treatment in vitro and to hypercholesterolemic stress in animal models of atherosclerosis in vivo. Human endothelial cells in culture express E-selectin, ICAM-1, and VCAM-1 at high levels when stimulated with cytokines, and this effect is attenuated by E2 treatment. In vitro evidence suggests that the effect of E2 on adhesion molecules is mediated by inhibition of nuclear factor (NF)-κB DNA binding, probably by stabilizing and increasing NF-κB inhibitors. In vivo studies support...
endoluminal injury, consistent with our previous reports of extensive estrogenic suppression of VCAM-1 as the most important relation among the adhesion molecules. OVX double-knockout mice with apoE<sup>−/−</sup> and either P-selectin<sup>−/−</sup> or ICAM-1<sup>−/−</sup> demonstrated atherosclerotic burdens similar to apoE<sup>−/−</sup> mice alone. In contrast, a 38% decrease in VCAM-1 expression was seen in E2-treated mice and was associated with a decrease in atherosclerosis burden. These results are supported by important estrogenic attenuation of VCAM-1 expression that coincided with less atherosclerosis formation in a hypercholesterolemic rabbit model. Our results demonstrate important in vivo increases in vascular production of VCAM-1, P-selectin, and ICAM-1 in response to endoluminal injury and marked global inhibition of this response by E2 within 2 hours of injury. The E2 effect dissipated by 24 hours, with only P-selectin remaining significantly suppressed by E2 treatment (Figure 1). Taken together with previous studies, our findings suggest that estrogenic suppression of adhesion molecule expression may be a significant component of the early antiinflammatory and vasoprotective effects of E2.

Consistent with our previous observations of extensive E2-inhibitable neutrophil infiltration of injured arteries, the most dramatic finding of the present study is marked overexpression of the selective neutrophil chemokine CINC-2, accompanied by increased neutrophil chemotaxis in injured arteries and E2 inhibition of both of these processes. As members of the CXC chemokine family, rat CINC-1, CINC-2α, CINC-2β, and CINC-3 are neutrophil-specific chemokines that bind to a common receptor (CXCR2), show structural and functional homology to human IL-8, and are potent neutrophil chemoattractants in vitro and in vivo. In a variety of acute vascular injury models, including a rat stroke model of middle cerebral artery occlusion with reperfusion and a rat liver transplant model with prolonged cold ischemia, upregulation of CINC has been demonstrated and correlated with neutrophil infiltration. These acute-injury responses may play important roles in many pathological conditions, including acute coronary syndromes, as demonstrated by experimental and autopsy histological studies that show neutrophil infiltration. An intervention that attenuates the processes that lead to acute neutrophil recruitment might have far-reaching vasoprotective implications.

The present study reveals marked estrogenic modulation of CINC mRNA and protein expression in the vasculature after endoluminal injury, consistent with our previous reports of attenuated neutrophil infiltration and of reduced neointima formation with E2 treatment. To our knowledge, this report is the first in vivo study of CINC or CXCR2 ligand regulation by E2. Previous in vitro studies have tested the effects of E2 on mRNA and protein expression of CXC chemokine growth-regulated oncogene α (GROα) and human IL-8 in vascular tissues. In one study, E2 treatment of human umbilical vein endothelial cells (HUVECs) markedly reduced basal GROα expression, and this effect was blocked by tamoxifen, suggesting estrogen receptor-α dependence. In a second study, E2 attenuated TNF-α–stimulated IL-8 mRNA and protein expression by 54% in HUVEC culture. Tamoxifen ablated the effect of E2. These studies offer indirect support to our hypothesis that E2 inhibits vascular expression of chemical mediators that guide neutrophil infiltration and are consistent with our previous demonstration that estrogenic vasoprotection in the vascular injury model is estrogen receptor dependent.

Our demonstration that extracts of homogenized, injured, carotid arteries from E2-treated rats produce less neutrophil chemotaxis than do those from V-treated rats is also consistent with previous in vitro evidence that E2 inhibits neutrophil chemotaxis via an estrogen receptor–dependent mechanism. In transwell migration assays, E2 treatment inhibited human neutrophil chemotaxis toward N-formylmethionyl-leucyl-phenylalanine-methylester in a dose-dependent manner, whereas pretreatment with clomiphene or tamoxifen eliminated the inhibitory effect of E2 on migration and restored neutrophil chemotaxis to control levels. Similarly, extracts of E2-treated HUVECs that had been stimulated with TNF-α expressed reduced levels of MCP-1 and IL-8 compared with untreated controls and produced less U-937 monocyte chemotaxis in a transwell migration assay than did extracts of untreated HUVECs. Measurement of neutrophil (and other leukocyte) chemotactic activity of extracts from injured vessels provides a useful bioassay of the effects of E2 and other potential modulators of inflammation in the setting of acute vascular injury.

Taken together, our in vivo and in vitro evidence supports the hypothesis that E2 produces vasoprotection, at least in part through negative modulation of neutrophil-specific chemokines. Together with our previous studies and those of others, these findings provide evidence for a pivotal role for neutrophils in the acute vascular injury response and suggest a novel mechanism by which E2 may inhibit neointima formation in this setting. Further mechanistic work is needed to elucidate the cellular and molecular pathways through which E2 mediates its antiinflammatory and vasoprotective actions.

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References


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