17β-Estradiol Attenuates Development of Angiotensin II–Induced Aortic Abdominal Aneurysm in Apolipoprotein E–Deficient Mice

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Objectives—Angiotensin II (Ang II) promotes vascular inflammation, accelerates atherosclerosis, and induces abdominal aortic aneurysm (AAA). These changes were associated with activation of nuclear factor (NF)-κB–mediated induction of proinflammatory genes. The incidence of AAA in this model was higher in male than in female mice, and the vascular effects of estrogen may be associated with anti-inflammatory actions. The present study was undertaken to test the hypothesis that estrogen can attenuate Ang II–induced AAA in apolipoprotein E–deficient mice via its anti-inflammatory mechanism.

Methods and Results—Infusion of Ang II (1.44 mg/kg per d for 1 month) induced AAA in 90% of the animals (n=20) with an expansion of the suprarenal aorta (diameter 1.9±0.14 mm versus <1 mm in normal mice). In mice treated with 17β-estradiol (E2, 0.25-mg subcutaneous pellets), Ang II induced AAA only in 42% of the animals (n=19) with a significant reduction of average diameters of the suprarenal aorta (1.5±0.14 mm). E2 also decreased the expressions of intracellular adhesion molecule-1, vascular cellular adhesion molecule-1, E-selectin, monocyte chemotactic protein-1, and macrophage-colony stimulating factor in the aorta.

Conclusions—These data suggest that attenuation of AAA by E2 is associated with inhibition of proinflammatory gene expression. (Arterioscler Thromb Vasc Biol. 2003;23:1627-1632.)

Key Words: estrogen □ aneurysm □ vascular inflammation □ PPAR □ gene expression

Atherosclerosis is an inflammatory disease. Infiltration of monocytes and macrophages into the vessel wall is a major source of proteolytic enzymes, including metalloproteinases, which degrade extracellular matrix, thus impairing the integrity of the artery wall and causing aneurysm. Angiotensin II (Ang II) has been implicated in vascular inflammation and progression of atherosclerosis. Chronic infusion of Ang II in apolipoprotein E–deficient (apoE-KO) mice induces aneurysm. We demonstrated that vascular inflammation and aneurysm induced by Ang II were associated with activation of nuclear transcription factor (NF)-κB–mediated proinflammatory gene induction.

It has been reported clinically that the incidence of abdominal aortic aneurysm (AAA) was higher in males than in females. Ang II–induced AAA was also higher in male than in female mice in this model. Estrogen has long been recognized to have vascular actions. Treatment with estrogen reduces atherosclerotic lesion formation in several animal models, including apoE-KO mice, even in the absence of lipid-lowering effects. Accumulating evidence suggests that the antiatherosclerotic effects of estrogen can be attributed, at least in part, to anti-inflammatory actions. Furthermore, there is in vivo evidence that estrogen inhibits the NF-κB pathway, which contributes to anti-inflammatory actions.

The present study, therefore, was aimed to test the hypothesis that treatment of 17β-estradiol can attenuate Ang II–induced AAA in apoE-KO mice via the mechanism of inhibiting NF-κB–mediated proinflammatory gene induction.

Methods

Male apoE-KO mice (Jackson Laboratory, Bar Harbor, Maine) were fed normal rodent chow. When the mice were 5 months old, a 17β-estradiol pellet (0.25 mg/pellet, 60-day release, IRA) was implanted subcutaneously (E2). A separate group of age-matched apoE-KO mice with no E2 implantation was used as control. According to the data provided by the manufacturer, this dose of E2 can reach blood concentration of 100 to 150 pg/mL, a level well within the physiological range in women before ovulation. After 30 days, when the animals were 6 months old, an osmotic mini-pump (Alzet, model 2004) containing Ang II (1.44 mg/kg per d) was implanted subcutaneously in both the E2 and control groups. Thirty days after mini-pump implantation, blood pressure and heart rate were measured noninvasively in conscious animals by the tail-cuff

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Blood samples were collected via cardiac puncture for the measurement of serum cholesterol profile (IDEXX Veterinary Services). In separate groups of age-matched apoE-KO mice treated with vehicle or Ang II with or without E2 as described above, total blood leukocyte count was determined using an automated hematology analyzer (Baker 9120, BioChem ImmunoSystems Inc.).

The outer diameter of the suprarenal region was measured from the image captured by a calibrated digital camera attached to a microdissecting microscope as described in detail previously. The severity of the aneurysms was subjectively accessed using a 4-grade scoring system based on gross appearance of the aorta as described in detail by Daugherty et al. Criteria were as follows: type 0 indicated no aneurysm, with the suprarenal region of the aorta not obviously different from naive apoE-KO mice without Ang II treatment; type I, dilated lumen with no thrombus; type II, remodelled tissue that frequently contained thrombus; type III, a pronounced bulbous form of type II that contained thrombus; and type IV, a form in which there were multiple aneurysms containing thrombus.

The left and right carotid arteries were dissected for quantification of atherosclerotic lesion area as described in detail previously and in the online data supplement (available at http://atvb.ahajournals.org). A transverse section of the suprarenal aorta was excised for immunostaining with an antimacrophage antibody (MAC-3; BD Pharmingen) and counterstained with hematoxylin. Rabbit IgG was used as a negative control (Sigma Chemical). To determine the extent of elastolysis and collagen content, adjacent cross-sections of the same aorta were stained with H&E, elastin-Van Gieszen, and trichrome.

As described in detail previously and in the online data supplement, expressions of aortic mRNAs for intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), endothelial-selectin (E-selectin), monocyte chemotactic protein-1 (MCP-1), macrophage-colony stimulating factor (M-CSF), PPAR-α, and PPAR-γ were determined by real-time polymerase chain reaction. NF-κB activity was measured by electrophoretic mobility shift assay from the nuclear extracts of aortic tissue.

Nuclear extracts isolated from individual aorta were pooled as 2 samples: control (n=5) and E2 (n=5). The aortic nuclear extracts from an additional 5 age-matched naive apoE-KO mice without any treatment were used for the measurement of baseline activities of the transcription factors. Using the Myria protein/DNA array kit (Panomics), a set of biotin-labeled DNA-binding oligonucleotides were preincubated with aortic nuclear extracts to allow the formation of DNA/protein complexes. The DNA/protein complexes were then extracted and hybridized to the MYRIA array that contained 54 consensus-binding sequences corresponding to a specific eukaryotic transcription factor. Detection of signals was quantified using the Kodak Electrophoresis Documentation & Analysis System 290 (Eastman Kodak) and normalized to biotinylated DNA spotted on each array. A percent change of activated transcription factors over baseline was compared between the Ang II–infused apoE-KO mice treated with (E2) or without estrogen (control). All experiments were performed in duplicate.

Results

Treatment of E2 significantly reduced atherosclerotic lesion area in the carotid arteries by 75% compared with controls (Figure 1, top). Systolic blood pressure was not significantly different between the 2 groups (Figure 1, bottom). There were no significant differences in body weight or cholesterol profiles between the control and E2 groups (see online Table 1). Ang II treatment significantly decreased the circulating leukocytes (7450±1088 per μL, n=10) compared with the mice treated with vehicle only (12040±1254 per μL, n=10). Combination of

E2 with Ang II additionally decreased circulating leukocytes to 4688±468 per μL (n=8), which, however, is not statistically different from mice treated with Ang II only.

Figure 1. Atherosclerotic lesion area (top) and systolic blood pressure (bottom) in Ang II-infused apoE-deficient mice treated with (E2) or without (control) 17β-estradiol.

17β-Estradiol Attenuated Angiotensin II–Induced Aneurysm Formation in ApoE-KO Mice

In the control group, infusion of Ang II induced AAA in 90% of the apoE-KO mice (n=20). Treatment with E2 significantly reduced Ang II–induced AAA to 42% (n=19). Consequently, the average outer diameter of the suprarenal aorta was significantly smaller in the E2 than control group (Figure 2, top). In the E2-treated mice that still developed AAA, the severity (category score) of the aneurysm appeared to be less than in the control group (Figure 2, bottom).

Figure 2. Average diameters of the suprarenal aorta (top) and classification of aneurysm type (bottom) in angiotensin II–infused apoE-deficient mice treated with (E2) or without (control) 17β-estradiol.
Histological evaluation of the arteries from naive mice showed intact endothelium overlying an intact internal elastic lamina (Figure 3). The modest media had little collagen and was invested with concentric layers of elastin. The adventitia was relatively thin and composed of woven bands of collagen. Control mice treated with Ang II had thick walls with intimal plaques, irregular media, and prominent adventitia. The intima was occasionally disrupted by plaques of Mac-3–positive foam cells on the luminal side of the internal elastic lamina. The thickness of the media was increased by extracellular matrix that was deposited between smooth muscle bundles and stained with trichrome as collagen. Elastin fibers in the media were discontinuous and irregularly oriented (Figure 3 and online Figure I). The adventitia was markedly thickened by extracellular matrix that was predominately collagen. A modest increase in adventitial cellularity included fibroblasts and Mac-3–positive mononuclear cells. Segmental regions of arterial wall had replacement of the media and adventitia by collagenous matrix. Aortas from E2-treated animals were thicker than naive animals but had fewer intimal plaques and no evidence of macrophage accumulation in the intima. These vessels did have medial changes including collagen deposition and some increase in elastin fibers, but the internal elastic lamina generally remained intact and retained a distinctive media of smooth muscle. The adventitia was thickened by collagen and Mac-3–positive cells to an extent similar to that in the control animals.

**17β-Estradiol Decreased NF-κB–Mediated Proinflammatory Gene Expression and Upregulated PPAR Gene Expression in the Aortic Wall**

Electrophoretic mobility shift assay detected a 31% decrease in NF-κB activity in the E2 group compared with that of the control group (Figure 4, top and middle). However, it did not reach statistical significance. DNA/protein binding array detected a 27% to 60% increase in transcription factor activation, including activator protein-2 (AP-2), Ets, murine embryonic fibroblast-1 (Mef-1), c-Myb, early growth response protein (Egr), NF-E2, specificity protein-1 (Sp-1), and cAMP-responsive element binding protein (CREB) in the control apoE-KO mice only treated with Ang II compared with the age-matched naive apoE-KO mice without any treatment (Figure 4, bottom). Treatment with E2 reversed the stimulatory effects of Ang II on these transcription factors, which was accompanied by a significant decrease in the expression of proinflammatory genes, including ICAM-1, VCAM-1, E-selectin, MCP-1, and M-CSF in the
suprarenal aorta (Figure 5, top), where aneurysm was consistently localized, as well as in the other regions of the aorta (Figure 5, bottom). In contrast, the expression of both PPAR-α and PPAR-γ was significantly increased in all sections of the aorta from the E2 group compared with that from the controls (online Figure II).

Discussion

The present results demonstrated that treatment of estradiol significantly reduced the incidence and severity of Ang II–induced AAA and atherosclerosis in apoE-KO mice. These effects were associated with a downregulation of the expression of several NF-κB–dependent proinflammatory mediators, including ICAM-1, VCAM-1, E-selectin, MCP-1, and M-CSF in the aorta (bottom) from the angiotensin II–infused apoE-KO mice pretreated with (E2) or without (control) 17β-estradiol. P<0.05 between the 2 groups.

Our previous study demonstrated that treatment of Ang II in apoE-KO mice upregulated the expression of several proinflammatory genes in the aortic wall, including ICAM-1, VCAM-1, E-selectin, MCP-1, and M-CSF.6 The induction of these proinflammatory genes is mediated predominantly by the activation of the NF-κB signaling pathway.20 The transcriptional regulation of NF-κB involves physical interaction with other transcription factors, such as Sp-1, CREB, and Egr-1.21 Many stimuli associated with the development of vascular disease, including Ang II, are capable of inducing these transcription factors.3,22,23 Once activated, the transcription factors NF-κB, Egr-1, and Sp-1 bind to recognition elements in the promoter region of proinflammatory genes and act as dominant regulators of transcription of these genes to induce inflammation.24

Estrogen has long been recognized, although controversially, as a cardiovascular protective agent.10 In many studies, estrogen has been shown to reduce atheroma formation.11,12,25 The present study demonstrated that treatment of E2 significantly reduced atherosclerotic lesions in apoE-KO mice infused with Ang II. It has been shown that estrogen inhibits monocyte adhesion, decreases VCAM-1 and MCP-1 expression in hypercholesterolemic rabbits,14,15 and reduces plasma levels of adhesion molecules and other markers of endothelial activation in postmenopausal women.26,27 Other studies have demonstrated that the inhibition of VCAM-1 expression by estrogen in cultured endothelial cells is through the interference of NF-κB activity,28 probably involving inhibition of DNA binding,29 induction of inhibitory κB (IκB), a natural inhibitor of NF-κB, or coactivator sharing.30,31 Furthermore, Evans et al30 demonstrated in vivo reciprocal antagonism between estrogen receptors and NF-κB activity, suggesting that the anti-inflammatory benefits of estrogen may be attributable to its ability to interfere with NF-κB–mediated inflammatory gene activation in the vasculature.

In support of this view, the present data demonstrated that attenuation of Ang II–induced AAA and atherosclerosis by E2 was associated with the inhibition of NF-κB, Sp-1, and Egr-1 activities and downregulation of proinflammatory genes. Using DNA/protein binding array technology, the present study also demonstrated that Ang II treatment in apoE-KO mice activated several transcription factors, including AP-2, Ets, Mef-1, Egr, NF-E2, Sp-1, and CREB. These transcription factors may be related to vascular inflammation, atherosclerosis, and aneurysm formation. For example, it has been shown that Egr may play a key regulatory role by linking injurious stimuli, including Ang II, to induce proinflammatory genes that ultimately result in vascular pathology.32,33 CREB has been shown to induce Ang II type 1 receptors, IL-6, COX-2, and VEGF, promote thrombin-induced smooth muscle proliferation, and function as a mediator of growth factor signals.34 In addition, Sp-1, Ets, Ap-1, and Sp-2 have been shown to induce uPA and its receptors and metalloproteinases,35,36 These matrix-degrading proteases are involved in aneurysm formation.2,7 Consistent with these reports, the present study also demonstrated that E2 treatment inhibited the activities of the above transcription factors, sug-
suggesting that this mechanism may also contribute to the effects of E2 in attenuating atherosclerosis development and AAA formation in this model. Accumulating evidence suggests that estrogen directly inhibits the growth-promoting effects of renin-angiotensin system in vascular smooth muscle cells. This mechanism could also contribute to the protective effects of estrogen on neointima formation in response to Ang II.

The cardiovascular protective effects of estrogen also include several other aspects. For example, estrogen has been shown to decrease blood pressure in hypertensive animals. Although blood pressure changes could contribute to AAA formation, it was not the case in the present study because blood pressure was not significantly different between the control and E2 groups. It is interesting that the increased expression of proinflammatory genes by Ang II reported in our previous study and the suppressive effect of E2 on the expression of these genes in the present study did not vary between different regions of the aorta. However, aneurysm is consistently localized in the suprarenal region in this model. Although the exact mechanism is still unclear, we speculate that when blood flow passes from the thoracic aorta (with negative pressure extravascularly) to the abdominal aorta (with positive pressure extravascularly), the flow becomes more turbulent in the suprarenal region, thus causing more stress damage to the endothelium, which facilities more inflammatory cell filtration into this region. Estrogen also exerts favorable changes in the cholesterol profile. This, too, cannot be the explanation for the effects of E2 in reducing Ang II–induced AAA and atherosclerosis in the present study, because cholesterol levels were not different from the control groups.

Histological examination revealed that there were fewer intimal plaques and relatively fewer macrophages, at least qualitatively, in the intima of the aortic wall in mice treated with E2. Reduction of arterial expression of proinflammatory genes by estrogen could inhibit inflammatory cell migration into the vascular wall. On the other hand, decreased arterial expression of proinflammatory genes and transcription factors could also be a consequence of decreased number or density of inflammatory cells. The present data cannot differential such cause and effect consequence. Our data also showed that circulating leukocytes were approximately 37% lower in mice treated with Ang II plus E2 compared with those receiving Ang II only. However, the reduction in tissue macrophage by E2 is most likely a consequence of its effect in preventing endothelial activation and local production of chemoattractants. This view is supported by a report by Alvarez et al, who provided direct in vivo evidence that estrogen inhibits Ang II–induced leukocyte–endothelial cell interactions. Circulating leukocytes maintain their integrins in nonadhesive states, whose avidity to endothelial ligands is stimulated by endothelium-displayed chemokines. Our data that Ang II alone reduced circulating leukocytes by approximately 37% despite its effects in promotion of inflammatory cell migration also support the view that circulating levels of leukocytes may not directly affect their capability to migrate into the vascular wall. This is in agreement with the report by Panzer et al, in which the glomerular monocyte infiltration occurred despite a 50% reduction in the circulating leukocytes in an immune-mediated mesangial cell injury model.

PPARs are members of the nuclear receptor superfamily of transcription factors that controls the expression of a large array of genes. Experimental data indicate that both PPAR-α and -γ play an important role in modulating vascular inflammation. In support of this view is the observation that inflammation, provoked by arachidonic acid or its derivative leukotriene B4 (LTB4), is prolonged in PPAR-α–deficient mice. Many of the anti-inflammatory responses of PPARs may result from the inhibition of the NF-κB signaling pathway. This view is reinforced by a recent discovery that the anti-inflammatory prostaglandin, 15-deoxy-Δ12,14-prostaglandin J2, also a natural PPAR-γ ligand, directly inhibits IκB kinase, which inactivates IκB by phosphorylation. Furthermore, our previous study demonstrated that Ang II–induced AAA in apoE-KO mice was associated with a significant downregulation of both PPAR-α and -γ expression in the aorta, thus impairing an important anti-inflammatory defense mechanism. Diminished PPAR expression may contribute, at least in part, to Ang II–induced vascular inflammation. Treatment with PPAR agonists in Ang II–infused apoE-KO mice dramatically decreased NF-κB, Egr-1, and Sp-1 transcriptional activation and downregulated proinflammatory gene expression. Thus, the upregulation of PPAR after E2 treatment in the present study may contribute to the vasculoprotective effects of E2 through attenuation of NF-κB–mediated proinflammatory gene expression.

In summary, the present data indicate a broad array of cellular actions for the anti-inflammatory and vasculoprotective actions of estrogen. Attenuation of atherosclerosis and AAA by estrogen could result from its inhibition of arterial expression of proinflammatory genes and transcription factors, which consequently reduced inflammatory cell migration into the vascular wall.

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