Inhibitory Effects of AT₁ Receptor Blocker, Olmesartan, and Estrogen on Atherosclerosis Via Anti-Oxidative Stress

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Abstracts—The present study explored the possibility that estrogen enhances the inhibitory effect of an angiotensin II type-1 (AT₁) receptor blocker (ARB), olmesartan, on atherosclerosis, focusing on oxidative stress using apolipoprotein E knockout mice (ApoEKO). After 6 weeks on a high-cholesterol diet, marked atherosclerotic lesion formation with an increase in oxidative stress, such as superoxide production, NAD(P)H oxidase activity and expression of p47phox mRNA and rac-1 mRNA, were observed in the proximal aorta in both male and female ApoEKO mice, whereas these changes were less marked in female mice. Ovariectomy enhanced these parameters, the changes of which were reversed by 17β-estradiol (80 μg/kg per day) replacement. Treatment with olmesartan (3 mg/kg per day) significantly inhibited oxidative stress and atherosclerosis, whereas its inhibitory effects were more marked in female than in male or ovariectomized mice. Smaller doses of olmesartan (0.5 mg/kg per day) or 17β-estradiol (20 μg/kg per day) did not influence atherosclerosis and oxidative stress in ovariectomized mice, whereas co-administration of olmesartan and 17β-estradiol at these doses attenuated these parameters. An angiotensin-converting enzyme (ACE) inhibitor, temocapril, also inhibited atherosclerotic changes similarly to olmesartan. Moreover, angiotensin II–mediated activation of NAD(P)H oxidase in cultured vascular smooth muscle cells was attenuated by 17β-estradiol. These results indicate that estrogen and an ARB synergistically attenuate atherosclerosis at least partly via inhibition of oxidative stress.

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Key Words: atherosclerosis ■ estrogen ■ oxidative stress ■ receptors, angiotensin II

Well-established cardiovascular risk factors, such as dyslipidemia, hypertension, diabetes, and smoking, can initiate endothelial dysfunction by altering the cell redox state in the vessel wall and induce atherosclerosis. In this paradigm, a common mechanism by which cardiovascular risk factors initiate the disease process is oxidative stress, leading to vascular inflammation. Excessive production of reactive oxygen species has been implicated in many pathophysiological conditions such as oxidative stress. Reactive oxygen species are involved in hypertension, proliferation, and migration of vascular smooth muscle cells (VSMCs) and matrix regulation,1–3 and cause hypertension, atherosclerosis, restenosis, and diabetes mellitus vasculopathy.4 NAD(P)H oxidase produces superoxide anion, one of the important reactive oxygen species, by catalyzing 1-electron reduction of oxygen using NAD(P)H as the electron donor.5 NAD(P)H oxidase consists of membrane components, nox1, nox4, and p22phox, and cytoplasmic components, p47phox and rac-1.6

There is evidence that angiotensin (Ang) II is more than a hormone with hemodynamic actions. It is also a local, biologically active mediator that plays a key role in the initiation and amplification of pathobiological events that lead to vascular disease.7 Ang II is a major mediator of oxidative stress by activating NADH/NAD(P)H oxidase via the Ang II type 1 (AT₁) receptor, which results in production of the superoxide anion.8,9 However, epidemiological and clinical evidence suggests that estrogen shows protective effects against cardiovascular disease,10–12 although their mechanisms are incompletely understood. Estrogen replacement therapy suppresses the incidence of cardiovascular disease in postmenopausal women,11 and it reduces plasma low-density lipoprotein cholesterol and increases high-density lipoprotein cholesterol levels.13 However, the alterations in lipid profile reported account for only a limited portion of the protective effect of estrogen against cardiovascular disease.

The major cardiovascular actions of Ang II have been reported to be mediated by the AT₁ receptor, and AT₁ receptor blockers (ARBs) have been widely used as antihypertensive drugs with the expectation of a vascular protective effect. These results led us to explore the possibility that the vascular protective effects of ARBs would be at least partially caused...
by exaggeration of the inhibitory effect of estrogen on oxidative stress in atherosclerosis using a mouse atherosclerosis model with apolipoprotein E knockout mice (ApoEKO), in which lesions similar to those observed in humans develop. Moreover, we examined the possibility that estrogen could inhibit AT1 receptor–mediated NADH/NAD(P)H oxidase activation via direct signaling cross-talk of estrogen and Ang II in cultured VSMCs.

**Methods**

**Animals and Treatment**

Adult ApoEKO mice (The Jackson Laboratory, Bar Harbor, Me) aged 6 weeks were given a standard diet or high-cholesterol diet (HCD) (1.25% cholesterol, 10% coconut oil) for 6 weeks. Ovariectomy was performed 2 days before feeding the HCD. The Animal Studies Committee of Ehime University approved the experimental protocol. 17β-Estradiol (20 and 80 μg/kg per day) was injected subcutaneously.In. Olmesartan (RNH-6270; donated by Sankyo Pharmaceutical Co, Tokyo, Japan), a specific AT1 receptor blocker, was administered intraperitoneally using an osmotic minipump at a dose of 0.5 and 3 mg/kg per day for 14 days. Temocapril, an angiotensin-converting enzyme (ACE) inhibitor (0.5 and 1.0 mg/kg per day; donated by Sankyo Pharmaceutical Co, Tokyo, Japan), was administered by gavage for 14 days. Plasma cholesterol level was measured as previously described. The mice were euthanized at 12 weeks of age, and microscopic quantitative analysis of atherosclerotic lesions were performed using cross-section of aorta as previously described.16

**Measurement of Superoxide**

Histological detection of superoxide anion was performed as described previously.17

**NAD(P)H Oxidase Activity**

Tissue protein sample was prepared from the intact endothelium of aortic arch after homogenization in 500 μL ice-cold Tris-sucrose buffer. NAD(P)H oxidase activity was quantified with cytochrome c method from the absorbance with or without SOD as previously described.18 NAD(P)H oxidase activity in cultured VSMCs was measured using lucigenin as previously described.19

**Real-Time Reverse-Transcription Polymerase Chain Reaction**

Real-time quantitative reverse-transcription polymerase chain reaction (PCR) was performed with a SYBR green I kit (MJ Research, Inc, Waltham, Mass). PCR primers for p47phox were 5'-TCATCACTGCTGCCCTTGAG-3' (forward) and 5'-CTCGCTACTTGCTCTGGTGA-3' (reverse); for AT1 receptor, 5'-GGGACATCTGCAGCAGTTCG CAGG-3' (forward) and 5'-CATCAGGATGATGAA TGGTCA-3' (reverse); for rac-1, 5'-GACATCGTGTATGC CCTTTGAC-3' (forward) and 5'-GACATCGTGTATGCCTTTGAC-3' (reverse); and for β-actin, 5'-GGGACATCTGCAGCAGTTCG CAGG-3' (forward) and 5'-GGGACATCTGCAGCAGTTCG CAGG-3' (reverse).

**Tissue Ang II Concentration**

Protein sample was prepared from aorta, and the concentration of Ang II in aortic tissue was measured using EIA kit as previously described.20

**Membrane Preparations and Scatchard Plots**

Membrane fraction of aorta was prepared and Scatchard plot analysis was performed using 2.6 pg/mL 125I-[Sar1, Ile8] Ang II (NEN Life Science Products, Inc, Boston, Mass) as previously described.20

**Culture of VSMCs and Receptor Binding Assay**

Adult rat aortic VSMCs were prepared and cultured as previously described.22 AT1 or AT2 receptor binding was measured as previously described.22

**Membrane Preparations in Cultured VSMCs and Western Blot Analysis**

The membrane fraction of VSMCs was prepared and immunoblotting of rac-1 was performed using anti-rac1 antibodies (Upstate Biotechnology Inc, Waltham, Mass) as previously described.20

**Statistical Analysis**

Values are expressed as mean±SE in the text and figures. The data were analyzed using 2-way ANOVA. If significance was found, Newman–Keuls test was performed for post-hoc analysis to detect the difference among groups. Values of P<0.05 were considered to be statistically significant.

**Results**

**Sex Difference in Atherosclerotic Lesions in Apo E-Deficient Mice Fed High-Cholesterol Diet**

Atherosclerotic lesion formation and lipid deposition, as well as plasma cholesterol level, were markedly increased in ApoEKO mice after HCD compared with those mice with a normal diet (Table I, available at http://www.hypertensiona-ha.org.). However, atherosclerotic changes were less marked in female mice (Figures 1 and 2 and Figure I). Plasma estrogen concentration decreased in ovariectomized (OVX) mice (54.6±2.6 pg/mL) compared with that in intact female mice (224.6±16.8 pg/mL), and it is similar to that in male mice (55.0±5.1 pg/mL). Ovariectomy enhanced lipid deposition and atherosclerotic lesion formation, which were recovered by administration of 17β-estradiol (80 μg/kg per day) (Figure 2 and Figure I). The lower dose of 17β-estradiol (20 μg/kg per day) did not significantly influence these parameters (Figure 2 and Figure I). OVX and estrogen treatment did not affect systolic blood pressure and heart rate (data not shown). Plasma estrogen concentration was 85.5±2.8 pg/mL and 193.3±3.7 pg/mL 14 days after administration of estrogen at doses of 20 and 80 μg/kg per day, respectively. Plasma cholesterol level was not significantly changed among male, female, and OVX mice or estrogen replacement (Tables I and II). In addition, the Ang II concentration in atherosclerotic aorta was not significantly changed by ovariectomy and 17β-estradiol replacement (57.0±7.0 pg/mg protein for ovariectomy, 58.0±1.0 pg/mg protein for ovariectomy with 17β-estradiol versus 59.0±5.0 pg/mg protein for intact female, respectively).

**Effect of Olmesartan on Atherosclerosis With Estrogen Treatment**

Olmesartan at 0.5 and 3 mg/kg per day did not affect systolic arterial pressure, heart rate, and plasma cholesterol concentration (data not shown). Olmesartan at 3 mg/kg per day significantly decreased lipid deposition and atherosclerotic lesion formation in the aorta of male, female, and OVX mice, whereas olmesartan at 0.5 mg/kg per day lowered these parameters only in female mice (Figure I). Olmesartan and 17β-estradiol at lower doses (0.5 mg/kg per day and 20 μg/kg per day, respectively) did not affect lipid deposition and atherosclerotic lesion formation in OVX mice, whereas co-
administration of both olmesartan and 17β-estradiol at these doses significantly decreased these parameters (Figure 3 and Figure II). Similar inhibitory action and combination effects occur with 17β-estradiol and nonhypotensive dose of an ACE inhibitor, temocapril (Figure III).

**Effect of Olmesartan and Estrogen on Oxidative stress in Atherosclerotic Lesion in ApoEKO Mice With HCD**

The chemiluminescent signal caused by superoxide production, tissue NAD(P)H oxidase activity, and expression of NAD(P)H oxidase subunit p47phox mRNA and rac-1 mRNA in the aorta assessed by quantitative real-time PCR were markedly enhanced in the aorta of ApoEKO mice maintained on HCD compared with ApoEKO mice maintained on normal diet, and were significantly higher in male and OVX ApoEKO mice than in female ApoEKO mice (Figures 4 and 5, the amplification curves of real-time PCR for p47phox are shown in Figure IV). These changes in OVX mice were attenuated by 17β-estradiol replacement (80 μg/kg per day). Olmesartan (3 mg/kg per day) significantly inhibited superoxide production, NAD(P)H oxidase activity, and expression of p47phox mRNA and rac-1 mRNA in male, female, and OVX mice, whereas this inhibitory effect of olmesartan was greater...
in female mice than in male and OVX mice (64.3% inhibition in female versus 11% inhibition in male mice; \( P < 0.05 \)) (Figures 4 and 5). Moreover, olmesartan at a lower dose (0.5 mg/kg per day) decreased these parameters only in female mice (Figures 4 and 5). Co-administration of 17\( \beta \)-estradiol and olmesartan at lower doses significantly inhibited these parameters in OVX mice (Figures 4 and 5).

The increase in AT\(_1\) receptor mRNA level in proximal aorta was more marked in male and OVX mice than in female ApoEKO mice with HCD, and AT\(_1\) receptor expression was decreased by 17\( \beta \)-estradiol (80 \( \mu \)g/kg per day) but not by olmesartan (Figure 5). In addition, the binding affinity of Ang II is not influenced by 17\( \beta \)-estradiol (Kd: 0.38 nmol/L for ovariectomy, 0.41 nmol/L for ovariectomy with 17\( \beta \)-estradiol replacement versus 0.40 nmol/L for intact female, respectively), but maximum binding of Ang II was significantly increased in OVX than in intact female (392 pmol/mg protein for OVX versus 250 pmol/mg protein for intact female, respectively), and restored by 17\( \beta \)-estradiol (215 pmol/mg protein) in Scatchard plots analysis (Figure V).

**Effect of Estrogen on Ang II–Mediated NAD(P)H Oxidase Activation in Cultured VSMCs**

We examined the possibility that estrogen may inhibit AT\(_1\) receptor–mediated NAD(P)H oxidase activity in VSMCs. Ang II (10\(^{-7}\) mol/L) increased NAD(P)H oxidase activity time-dependently (Figure VI). This increase was suppressed within 5 minutes by addition of 17\( \beta \)-estradiol (10\(^{-8}\) mol/L). Moreover, the AT\(_1\) expression was not changed by treatment with 17\( \beta \)-estradiol (data not shown). We also observed that the rac-1 protein in membrane fraction of VSMCs was increased 10 minutes after stimulation with Ang II, and that this increase was suppressed by addition of 17\( \beta \)-estradiol (Figure VII).

**Discussion**

Consistent with a previous report\(^{27}\) that 17\( \beta \)-estradiol protects against atherosclerotic lesion formation in the ApoEKO mice, and that this can be only partially explained through effects on plasma lipoprotein levels, we demonstrated that lipid deposition, atherosclerotic lesion formation, superoxide production, NAD(P)H oxidase activity, and expression of p47\(^{phox}\) and rac-1 mRNA were exaggerated in male ApoEKO mice compared with those in female mice, and that ovariectomy increased atherosclerosis, which was reversed by 17\( \beta \)-estradiol replacement. An ACE inhibitor, temocapril, also reduced atherosclerotic lesion similarly to olmesartan (Figure III). Plasma cholesterol level was markedly increased by HCD but did not significantly differ among male, female, and OVX mice, and was not changed by olmesartan or estrogen. Moreover, the combination of 17\( \beta \)-estradiol and AT\(_1\) receptor blockade induced a synergistic inhibition of atherosclerosis even at lower doses, which did not affect aortic lesion.

Superoxide generation and upregulation of NAD(P)H oxidase play an essential role in atherosclerotic lesion formation,\(^{28}\) and Ang II is a major mediator of oxidative stress by activating NAD(P)H oxidase via AT\(_1\) receptor.\(^{8,9}\) Estrogen has been reported to interfere with the renin-angiotensin system.\(^{30}\) Estrogen treatment leads to downregulation of renin and ACE, with a consequent reduction of Ang II production.\(^{30}\) Moreover, treatment with estrogen has been shown to downregulate AT\(_1\) receptor expression in aortic tissue and cultured VSMCs.\(^{31}\) We also reported that 17\( \beta \)-estradiol at-
tempered AT1 receptor–mediated activation of extracellular signal-regulated kinases and c-fos expression, thereby inhibiting VSMC proliferation. In the present study, the inhibitory effects of olmesartan on atherosclerosis and oxidative stress were stronger in female than in male or OVX mice. Moreover, we demonstrated that co-administration of olmesartan and 17β-estradiol even at lower doses synergistically inhibited lipid deposition and atherosclerotic lesion forma-

**Figure 4.** Effect of olmesartan and estrogen on superoxide production and NAD(P)H oxidase activity in ApoEKO mice. Freshly frozen sections of proximal aorta were prepared and superoxide was detected with dihydroethidium as described in Methods. Protein samples were prepared and NAD(P)H oxidase activity was measured as described in Methods. A and B, Representative detection of superoxide with dihydroethidium in cross-sections of proximal aorta. C, NAD(P)H oxidase activity in aorta of ApoEKO mice treated with HCD. Values are mean±SE of 7 to 8 experiments. E, 17β-estradiol. *P<0.05 vs ND, †P<0.01 vs HCD.

**Figure 5.** Effect of olmesartan and estrogen on expression of p47phox, rac-1, and AT1 receptor in ApoEKO mice. Aortic samples were taken from ApoEKO mice as described in Figure 1. Levels of mRNA for p47phox, rac-1, and AT1 receptor mRNA were assayed by quantitative real-time reverse-transcription PCR as described in Methods. A, Effect of olmesartan on expression of p47phox, rac-1, and AT1 receptor mRNA in male and female mice. B, Effect of combination of olmesartan and estrogen on expression of p47phox, rac-1, and AT1 receptor mRNA in ovariectomized female mice. Values are mean±SE of 5 experiments. *P<0.05 vs ND, †P<0.05 vs HCD, **P<0.05 vs HCD+OVX.
tion, superoxide production, NAD(P)H oxidase activity, and expression of p47phox mRNA and rac-1 mRNA in OVX mice. It has also been reported that NAD(P)H oxidase requires the p47phox and rac-1 components, which mediate superoxide release from VSMCs in the blood vessel wall in response to Ang II.22,31 These results suggest that estrogen would contribute at least partly to the ARB-mediated antiatherosclerotic effect via inhibition of oxidative stress, and that signaling cross-talk of the AT1 receptor and estrogen is important in the pathogenesis of vascular diseases.

Because the increase in AT1 receptor mRNA level was lower in female mice, and because it was decreased by 17β-estradiol (80 μg/kg per day) in OVX mice, it is suggested that downregulation of AT1 receptor mRNA expression by estrogen could contribute to the anti-atherogenic effect of estrogen. Scatchard plots analysis also indicated that the maximal binding of Ang II was increased in OVX mice, and this increase was recovered by 17β-estradiol replacement (Figure V). However, we observed that the inhibitory action of co-administration of olmesartan and 17β-estradiol was not dependent on AT1 receptor expression.

Gallagher et al suggested that estrogen lowers tissue levels of Ang II.34 In the present study, however, Ang II formation in the aorta was not significantly affected by ovariectomy or 17β-estradiol replacement. These results raised the possibility that estrogen directly inhibits Ang II–mediated NAD(P)H oxidase activation, and we observed that Ang II (10−7 mol/L) increased NAD(P)H oxidase activity time-dependently, and 17β-estradiol (10−8 mol/L) suppressed Ang II–mediated NAD(P)H oxidase activity without significant change in AT1 receptor binding. Moreover, 17β-estradiol suppressed Ang II–mediated translocation of rac-1 to the plasma membrane within 10 minutes (Figure VII). Because translocation of cytosolic component, such as rac-1, to the plasma membrane is necessary to activate NAD(P)H oxidase,35 our results suggest that estrogen could directly attenuate Ang II–mediated NAD(P)H oxidase activation at least partially by a nongenomic mechanism in cultured VSMCs.

Perspectives

In the present study, we showed that estrogen and ARBs in nonhypotensive doses synergistically improve atherosclerosis without changing plasma cholesterol level, accompanied by marked inhibition of oxidative stress. These observations provide new insights into the negative cross-talk between the actions of estrogen and Ang II in vivo. Moreover, we previously reported that estrogen and olmesartan synergistically attenuate vascular remodeling in a polyethylene cuff-induced vascular injury mouse model, which is at least partly via inhibition of extracellular signal-regulated kinases and signal transducer and activator of transcription activity.15 These results support the notion that combined, ARBs and estrogen replacement might be a useful and effective therapy for the treatment of cardiovascular disease associated with the menopause.

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References


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