Estrogen Activates Phosphatases and Antagonizes Growth-Promoting Effect of Angiotensin II

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Abstract—Accumulating evidence suggests that estrogen exerts cardioprotective effects and protects against neointima formation in response to vascular injury in vivo, whereas angiotensin (Ang) II stimulation via the Ang II type 1 (AT₁) receptor exaggerates vascular injury. We postulate that estrogen treatment antagonizes the AT₁ receptor–mediated growth-promoting effects in vascular smooth muscle cells (VSMCs). The present in vitro study was designed to explore this possibility and to establish the cellular mechanism whereby estrogen attenuates the growth of VSMCs. Primary cultures of VSMCs derived from male adult Sprague-Dawley rats express exclusively AT₁ receptors. Treatment with Ang II enhanced proliferation of VSMC and c-fos expression, whereas 17β-estradiol (E2) attenuated these vasotrophic effects of Ang II. We also demonstrated that E2 attenuated AT₁ receptor–mediated extracellular signal–regulated kinase activation and that this effect of E2 was restored by pretreatment with vanadate or okadaic acid. Moreover, we demonstrated that E2 enhanced SHP-1 activity, rapidly reaching a peak after 3 minutes of E2 stimulation, whereas E2 transactivated mitogen-activated protein kinase phosphatase-1 expression, showing a peak after 60 minutes of E2 treatment. SHP-1 activation was not influenced by actinomycin D treatment, whereas E2-mediated mitogen-activated protein kinase phosphatase-1 expression was attenuated. Taken together, our results suggest a novel mechanism of vasoprotection by which estrogen antagonizes the effect of the AT₁ receptor via the activation and induction of phosphatases through nongenomic as well as genomic signaling. (Hypertension. 2002;39:41-45.)

Key Words: angiotensin II ■ estrogen ■ receptors, angiotensin II ■ signal transduction ■ vasculature

One of the most highly recognized factors implicated in the pathogenesis of hypertension, atherosclerosis, congestive heart failure, and associated cardiovascular disease is angiotensin (Ang) II. Ang II has a significant influence on the heart and blood vessels via its effects on systemic hemodynamics and blood volume, and it also exerts long-term structural effects through its direct hypertrophic and proliferative actions. Moreover, epidemiological and clinical evidence suggests that estrogen is cardiovascular protective, and it is well known that estrogen retards the development of atherosclerosis; however, the mechanisms by which estrogen is cardiovascular protective are incompletely understood. Estrogen replacement therapy suppresses the incidence of cardiovascular disease in postmenopausal women and reduces plasma LDL cholesterol levels and increases HDL cholesterol levels. However, the alterations in lipid profile reported account for only a limited portion of the protective effect of estrogen against cardiovascular disease.

There is increasing evidence that estrogen interferes with the renin-angiotensin system. The beneficial cardiovascular effects of estrogen may be mediated in part by downregulation of ACE with a consequent reduction in the circulating level of the vasoconstrictor Ang II. Nickenig et al demonstrated that estradiol led to time-dependent downregulation of Ang II type 1 (AT₁) receptor mRNA in vascular smooth muscle cells (VSMCs). The major cardiovascular actions of Ang II have been reported to be mediated by a 7 membrane–spanning G protein–coupled receptor termed AT₁ receptor, which exerts vasoconstriction, aldosterone release, sodium and water retention, and cellular growth. Therefore, the cross-talk of Ang II with estrogen has been highlighted and antagonistic effects of Ang II and estrogen have been postulated; however, the molecular and cellular mechanisms of this interaction remain an enigma. We explored the possibility that estrogen may attenuate AT₁ receptor–mediated vasotrophic effects and examined the cellular and molecular mechanisms of potential cross-talk of Ang II and estrogen.

Methods

Cell Culture and Treatment

VSMCs were prepared from the thoracic aorta of male Sprague-Dawley rats (10 weeks old) and cultured in Dulbecco’s modified...
Eagle’s medium (DMEM; Life Technologies Inc) supplemented with 10% FBS (dextran coated and charcoal treated). To avoid the influence of the menstrual cycle on estrogen receptor expression, we used male rats. Subconfluent VSMCs were used in the following experiments. The medium was replaced with phenol red–free DMEM for 48 hours without serum to allow the cells to reach a quiescent state. Then, the cells were treated with phenol red–free DMEM containing Ang II (Sigma Chemical Co) and/or 17β-estradiol (Sigma Chemical Co) as indicated in each experimental condition. The Animal Studies Committee of Ehime University approved the experimental protocol.

Receptor Subtype Determination
AT₁ and Ang II type 2 (AT₂) receptor binding was measured as previously described.6,7

Measurement of DNA Synthesis
DNA synthesis was assayed by measuring [³H]thymidine incorporation as previously described.8

flos-Luciferase Assay
The flos-luciferase reporter vector (p2FTL) consists of 2 copies of the c-fos S²-regulated enhancer element (~357 to ~276), the herpes simplex virus thymidine kinase (TK) gene promoter (~200 to +7), and the luciferase gene.8 p2FTL (1 μg plasmid DNA) was transiently transfected into cultured VSMCs with LipofectAMINE PLUS (Life Technologies Inc) according to the manufacturer’s instructions (DNA/LipofectAMINE/PLUS ratio, 1 μg:5 μL:3 μL). After stimulation with Ang II or 17β-estradiol, or both agents, for 3.5 hours, luciferase activity was measured using cell lysates.

Extracellular Signal–Regulated Kinase Activity
Phosphorylation of extracellular signal–regulated kinase (ERK) was measured as previously described.8

Mitogen-Activated Protein Kinase Phosphatase-1 Expression
Time-dependent mitogen-activated protein kinase phosphatase-1 (MKP-1) expression was analyzed by Western blotting with anti-MKP-1–specific antibodies (1:500; Santa Cruz Biotechnology Inc).

Immunoprecipitation and Measurement of SHP-1 Tyrosine Phosphatase Activity
SHP-1 activity was measured as previously described.9–11

Plasmid Construct and Transfection
SHP-1 (C453/S) mutant cDNA was inserted into the pcDNA3 vector.10 Transient transfection was performed with 0.1 μg plasmid DNA per 24-well plate and LipofectAMINE PLUS (DNA/LipofectAMINE/PLUS ratio, 1:10:5).

Data Analysis
All values are expressed as mean±SEM. Data were evaluated by ANOVA followed by Newman-Keuls test for multiple comparisons. Differences with P<0.05 were considered to be significant.

Results
Antagonistic Effect of Estrogen on AT₁ Receptor–Mediated Cell Proliferation
To examine the role of endogenous AT₁ receptor in Ang II–regulated cell proliferation and its potential interaction with estrogen, we used VSMCs prepared from adult rat aorta. AT₁ receptor binding was 8.57±0.55 fmol/10⁶ cells, whereas no significant AT₂ receptor binding was observed, suggesting that VSMCs cultured from the male rat aorta exclusively express AT₁ receptor and minimal AT₂ receptor.

First, we investigated whether estrogen would influence AT₁ receptor–mediated VSMC growth. Cultured VSMCs were treated with Ang II or 17β-estradiol for 24 hours, and [³H]thymidine incorporation was examined. As shown in Figure 1, Ang II (0.3 μmol/L) increased [³H]thymidine incorporation into VSMCs. 17β-Estradiol did not affect [³H]thymidine incorporation into VSMCs, whereas 17β-estradiol attenuated the AT₁ receptor–mediated DNA synthesis dose-dependently. AT₁ receptor–mediated c-fos gene expression and the possible interaction of estrogen with AT₁ receptor were studied. The ability to induce c-fos gene expression was determined by measuring the increase in flos-luciferase activity in lysates after Ang II (0.3 μmol/L) and/or 17β-estradiol (0.1 μmol/L) treatment. As shown in Figure 2, Ang II stimulation resulted in a 12- to 13-fold increase in the expression of c-fos, whereas 17β-estradiol did not induce c-fos expression. In contrast, Ang II–induced c-fos expression was significantly attenuated by costimulation with 17β-estradiol in VSMCs. Moreover, we observed that treatment with 17β-estradiol for 48 hours did not significantly

Figure 1. Effect of estrogen on AT₁ receptor–mediated VSMC proliferation. Subconfluent VSMCs were incubated in phenol red– and serum-free DMEM for 48 hours and then treated with Ang II (0.3 μmol/L) or 17β-estradiol (E2; 0.1~ μmol/L) for 24 hours. DNA synthesis was assayed by measuring [³H]thymidine incorporation. Values are expressed as mean±SEM (n=4). Similar results were obtained in 3 different culture lines. *P<0.05 vs control.

Figure 2. Effect of estrogen receptor stimulation on the AT₁ receptor–mediated c-fos expression in VSMCs. p2FTL was transiently transfected into cultured VSMCs. Transfected cells were treated with Ang II (0.3 μmol/L) or 17β-estradiol (E2; 0.1 μmol/L) or both agents, and luciferase activity was determined. Values are expressed as mean±SEM (n=6). Similar results were obtained in 3 different culture lines. *P<0.01 vs control.
change AT1 and AT2 receptor binding. The density of AT1 receptor was 8.65 ± 0.43 fmol/10⁶ cells after 17β-estradiol treatment.

Interaction of AT1 Receptor and Estrogen Receptor on ERK
ERK mediates multiple cellular pathways, which are critical to cell proliferation, differentiation, and, in some cells, hypertrophy, and we observed that ERK is one of the critical determinants of AT1 receptor–mediated vasotrophic effects. As shown in Figure 3A, stimulation with Ang II activated ERK, and 17β-estradiol activated ERK to a lesser extent compared with Ang II. Interestingly, we observed that addition of 17β-estradiol to Ang II significantly inhibited AT1 receptor–mediated ERK activation (Figure 3A). Moreover, AT1 receptor–mediated ERK activation was further attenuated by pretreatment with 17β-estradiol for 60 minutes before Ang II addition.

Figure 3. Effects of estrogen receptor stimulation on AT1 receptor-mediated ERK activation in VSMC. A, top, Subconfluent VSMCs were incubated in phenol red– and serum-free DMEM for 12 hours and then stimulated with Ang II (0.3 μmol/L) or 17β-estradiol (E2; 0.1 μmol/L) or both agents for 15 minutes. Moreover, we pretreated VSMCs with 17β-estradiol for 60 minutes before Ang II addition. The cell lysate was subjected to immunoblotting for phospho-ERK. A, bottom, Densitometric measurements of phospho-ERK (ERK-1 and ERK-2). Values are expressed as mean ± SEM of 4 different pairs of culture lines. Subconfluent cells were treated with vanadate (10⁻⁵ mol/L) or okadaic acid (10⁻⁷ mol/L) in serum-free medium for 16 hours and then stimulated with Ang II (0.3 μmol/L) or 17β-estradiol (0.1 μmol/L) or both agents for 15 minutes. The cell lysate was subjected to immunoblotting for phospho-ERK. Values are expressed as mean ± SEM of 4 different pairs of culture lines.

Estrogen Activates a Variety of Phosphatases and Inhibits ERK Activation
We postulated that estrogen would activate and/or induce phosphatases, which deactivate the ERK activation cascade. VSMCs were treated with a tyrosine phosphatase inhibitor, vanadate (10 μmol/L), or a serine/threonine phosphatase inhibitor, okadaic acid (100 nmol/L), for 16 hours and stimulated with Ang II (0.3 μmol/L) and/or 17β-estradiol (0.1 μmol/L) for 15 minutes. As shown in Figures 3B and 3C, vanadate as well as okadaic acid attenuated the inhibitory effect of 17β-estradiol on AT1 receptor–mediated ERK activation, suggesting that protein tyrosine phosphatases and serine/threonine phosphatases are involved in these inhibitory effects of estrogen. The protein level of ERK was not affected by these treatments (data not shown).

We examined MKP-1 expression by Western blotting (Figure 4) and observed that 17β-estradiol increased the protein level of MKP-1 reaching a peak after 60 minutes of 17β-estradiol stimulation, and MKP-1 protein level then gradually decreased. This estrogen-mediated MKP-1 expression was inhibited by the addition of specific estrogen antagonist, ICI182780 (Astra-Zeneca) and by pretreatment with actinomycin D.

We demonstrated that 17β-estradiol activated SHP-1 (2 Src homology 2 [SH2] domain–containing cytosolic tyrosine phosphatase) activity after 3 minutes (Figure 5A), whereas AT1 receptor stimulation did not influence SHP-1 activity, as we previously reported. This estrogen-activated SHP-1 was inhibited by ICI182780, whereas actinomycin D did not influence the activation of SHP-1 (Figure 5B). To examine whether SHP-1 plays a role in estrogen receptor–mediated signaling, we transfected rat VSMCs with a dominant negative (dn) SHP-1 mutant in which the active site cysteine 453 was mutated to serine (C453S). Overexpression of the dn SHP-1 mutant was confirmed by immunoblotting showing a 6-fold increase in SHP-1 immunoreactivity compared with control vector pcDNA3-transfected cells. SHP-1 activation by 17β-estradiol was actually low in VSMCs transfected with dn SHP-1 mutant, and we could not observe a further significant increase in SHP-1 activity in response to 17β-estradiol (Figure 5C). Moreover, we observed that the inhibi-
Estradiol inhibited DNA synthesis, cellular proliferation, cell migration, collagen synthesis, and ERK in cultured human aortic VSMCs. The ERK cascade is inactivated at the level of members of the MKP family, including MKP-1, which have been suggested to provide a feedback loop to terminate growth factor signaling. Indeed, we observed that 17β-estradiol increased the protein level of MKP-1, and this estrogen-mediated MKP-1 expression was inhibited by pretreatment with actinomycin D, suggesting that the classic genomic estrogen signaling mechanism using cytoplasmic and nuclear estrogen receptors (ERs) is associated with new transcription of MKP-1. Consistent with this observation, Nuedling et al. also observed that estrogen induced MKP-1 in rat cardiomyocytes.

SHP-1 participates in negative regulation of the receptor tyrosine kinase pathway. For a number of membrane receptors such as the EGF receptor, SHP-1 terminates these receptors’ signals via direct interaction with these receptors or by deactivating the ERK cascade through undefined mechanisms. Moreover, we reported that cross-talk between the AT1 and AT2 receptors regulates the survival of fetal VSMCs and that SHP-1 is a key molecule in AT2 receptor signaling. Here, we demonstrated that 17β-estradiol activated SHP-1, rapidly reaching a peak at 3 minutes, and this SHP-1 activation was not influenced by actinomycin D treatment, suggesting that this estrogen-mediated SHP-1 activation was through membrane ERs. Estrogen is now believed to possess rapid membrane effects independent of the classic gene activation pathway of steroid action. The presence of membrane ERs has been demonstrated in different cell types but not yet in vascular tissue. Our results support the notion that SHP-1 is a critical determinant of ERK regulation by stimulation of surface ERs in VSMCs.

The new class of AT1 receptor blocker appears to provide cardiovascular protective effects; most of beneficial effects provided by AT1 receptor blockers appear to be related to more complete blockade of the AT1 receptor, and costimulation of the AT2 receptor appears to play some role in the improvement of cardiovascular remodeling. Moreover, specific AT1 receptor blockade may exaggerate the cardioprotective effects of estrogen. Thus, this combination of AT1 receptor blocker and estrogen replacement has the potential to be useful in the treatment of postmenopausal women with hypertension and consequent cardiovascular remodeling.

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