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Effect of Estrogen and AT\textsubscript{1} Receptor Blocker on Neointima Formation

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Abstract—The present study explored the possibility that estrogen may enhance the inhibitory effect of an angiotensin (Ang) II type 1 (AT\textsubscript{1}) receptor blocker on neointima formation in vascular injury, and investigated the signaling mechanism involved in their actions. Polyethylene cuff placement around the femoral artery of mice induced neointima formation and increased bromodeoxyuridine (BrdU) incorporation into vascular smooth muscle cells. These changes were significantly smaller in female mice than in male mice. Ovariectomy enhanced neointima formation and BrdU incorporation in the injured artery, which were reversed by 17\beta-estradiol (80 \( \mu \)g/kg per day) replacement. Treatment with a selective AT\textsubscript{1} receptor blocker, olmesartan (3 mg/kg per day), significantly inhibited neointima formation and BrdU incorporation, whereas the inhibitory effects of olmesartan were more marked in intact female mice than in male or ovariectomized mice. Phosphorylation of extracellular signal–regulated kinase (ERK), signal transducer and activator of transcription (STAT) 1, and STAT3 was increased in the injured artery. These increases were significantly smaller in intact female mice than in male or ovariectomized mice. Olmesartan or estrogen attenuated the phosphorylation of ERK and STAT in the injured artery, whereas these inhibitory effects were greater in intact female mice. Lower doses of olmesartan (0.5 mg/kg per day) or 17\beta-estradiol (20 \( \mu \)g/kg per day) did not influence neointima formation, BrdU incorporation, and ERK and STAT phosphorylation in ovariectomized mice, whereas coadministration of olmesartan and 17\beta-estradiol at these doses attenuated these parameters. These results indicate that estrogen and an AT\textsubscript{1} receptor blocker synergistically attenuate vascular remodeling, which is at least partly via inhibition of ERK and STAT activity. (Hypertension. 2002;40:451-457.)

Key Words: angiotensin \( \bullet \) estrogen \( \bullet \) kinase \( \bullet \) STAT \( \bullet \) vascular remodeling

Epidemiological and clinical evidence suggests that estrogen has protective effects on the cardiovascular system; however, their mechanisms are incompletely understood. Angiotensin (Ang) II has a significant influence on the heart and blood vessels via its effects on systemic hemodynamics and blood volume, and also exerts long-term structural effects through its direct hypertrophic and proliferative actions. The major cardiovascular actions of Ang II have been reported to be mediated by the Ang II type 1 (AT\textsubscript{1}) receptor, which mediates vasoconstriction, aldosterone release, sodium and water retention, and cellular growth. Estrogen has been reported to interfere with the renin-angiotensin system.\textsuperscript{1} The synthesis of angiotensinogen in hepatocytes is partially regulated by estrogen.\textsuperscript{2} Estrogen treatment leads to downregulation of renin and ACE, with a consequent reduction of Ang II production.\textsuperscript{3,4} Moreover, treatment with estrogen has been shown to downregulate AT\textsubscript{1} receptor expression in aortic tissue and cultured vascular smooth muscle cells (VSMCs).\textsuperscript{5} We postulate that estrogen treatment antagonizes the AT\textsubscript{1} receptor–mediated growth-promoting effects in VSMCs, and recently demonstrated that 17\beta-estradiol attenuated AT\textsubscript{1} receptor–mediated activation of extracellular signal-regulated kinases (ERKs) and c-fos expression, thereby inhibiting VSMC proliferation.\textsuperscript{6}

AT\textsubscript{1} receptor blockers (ARBs) constitute an important new class of antihypertensive drug, are widely used as antihypertensive treatment, and are considered to exert cardiovascular protective effects.\textsuperscript{7} Arterial neointimal thickening is an important process in the development of atherosclerosis, bypass graft failure, and restenosis after angioplasty. In injured arteries, components of renin-angiotensin system are upregulated, such as renin,\textsuperscript{8} angiotensinogen,\textsuperscript{9} ACE,\textsuperscript{10,11} and Ang II receptors.\textsuperscript{12,13} We have developed a mouse model of vascular disease induced by polyethylene cuff placement around the femoral artery, in which ACE and the AT\textsubscript{1} receptor are upregulated, followed by neointimal thickening.\textsuperscript{13} Estrogen replacement therapy suppresses the prevalence of cardiovascular disease in postmenopausal women\textsuperscript{14} and reduces plasma LDL cholesterol and increases HDL cholesterol levels.\textsuperscript{15} However, the alterations in lipid profile reported account for
only a limited portion of the protective effect of estrogen against cardiovascular disease. These results led us to explore the possibility that the vascular protective effects of ARB would be at least partially owing to exaggeration of the inhibitory effect of estrogen on VSMC proliferation in response to vascular injury in vivo.

Methods

Animals and Operation
Adult male and female C57BL/6J mice (Clea Japan Inc, Tokyo, Japan) were used in the present study. The mice were kept in a room in which lighting was controlled (12 hours on, 12 hours off), and the temperature was kept at 25°C. The mice were given standard diet (MF, Oriental Yeast Co Ltd) and water ad libitum. The Animal Studies Committee of Ehime University approved the following experimental protocol. The surgical procedure for cuff-induced vascular injury in the femoral artery was performed according to the methods described previously. Female mice underwent bilateral ovariectomy or sham-operation through a 1-cm abdominal incision at 11 weeks of age, as previously described, 1 week before cuff placement.

Treatment
17β-Estradiol (Sigma Chemical Co) was dissolved in propylene glycol (WAKO Chemical Inc). Ovariectomized (OVX) mice received a subcutaneous injection of 0.1 mL vehicle (propylene glycol) or 17β-estradiol daily from 1 day before cuff placement. Mice were killed by an overdose of anesthesia, and blood samples were collected from the inferior vena cava 14 days after injection. Plasma estrogen level was measured with an enzyme-linked immunosorbent assay kit (Cayman Chemical Co Inc). Olmesartan (RNH-6270, donated by Sankyo Pharmaceutical Co, Tokyo, Japan), a specific AT1 receptor blocker, was administered using an osmotic minipump (Model 1002, Alza Corp) implanted intraperitoneally at the same time as cuff placement, as previously described. The pump infused olmesartan continuously for 7 or 14 days at a rate of 0.25 μL/h.

Morphometric Analysis and Measurement of DNA Synthesis
Morphometric analysis and measurement of DNA synthesis were performed according to methods described previously.

Western Blot Analysis
Total protein was prepared from the pooled arteries after cuff placement (n=6 to 8 for each group), and Western blot was performed as previously described. Immunoblotting was performed using anti-ERK, anti-phospho-ERK, anti-signal transducers, and activators of transcription (STAT) 1, anti-phospho-STAT1, anti-phosphoSTAT3 (New England Biolabs), antiSTAT3 (Santa Cruz Biotechnology), and anti-α smooth muscle (α-SM) actin antibodies (clone 1A4; Sigma). Densitometric analysis was performed using an image scanner (EPSON GT-8000) and National Institutes of Health imaging software.

Data Analysis
Values are expressed as mean±SEM in the text and figures. The data were analyzed using ANOVA. If a statistically significant effect was found, post hoc analysis was performed to detect the difference between the groups. Values of P<0.05 were considered to be statistically significant.

Results

Effect of Estrogen on Neointima Formation
We compared morphological changes and DNA synthesis caused by polyethylene cuff placement in male and female mice. Cuff placement induced neointima formation in the femoral artery in both male and female mice, whereas neointimal thickening 14 days after the operation was significantly less in female mice than in male mice (330±224 μm² in female mice, 492±275 μm² in male mice; P<0.05) (Figure 1A). On the other hand, medial area was not significantly different between the experimental groups. We examined bromodeoxyuridine (BrdU) incorporation into VSMCs, as a marker of DNA synthesis, in the injured artery 7 days after cuff placement. Indexes of BrdU-positive cells in both the media and neointima were higher in male mice than in female mice (Figure 1B).

To examine the role of estrogen in neointima formation, female mice underwent ovariectomy. Plasma estrogen concentration decreased in OVX mice (40±18 pg/mL, n=5) compared with that in intact female mice (210±30 pg/mL, n=5), and there was no significant difference in plasma estrogen level between male (53±12 pg/mL, n=4) and OVX mice. Ovariectomy enhanced both neointima formation and incorporation of BrdU in the injured artery (Figure 1). There were no significant differences in these parameters between
male and OVX female mice. Administration of 17β-estradiol at a dose of 80 μg/kg per day significantly inhibited neointima formation and BrdU incorporation, whereas a lower dose of 17β-estradiol (20 μg/kg per day) did not significantly influence morphometric parameters and DNA synthesis (Figure 1). Plasma estrogen concentration was 185±45 pg/mL (n=5) and 85±15 pg/mL (n=5) 14 days after administration of estrogen at doses of 80 and 20 μg/kg per day, respectively. OVX and estrogen treatment did not affect systolic arterial pressure and heart rate (data not shown).

Effect of Olmesartan Plus Estrogen Treatment on Neointima Formation

To avoid the hemodynamic effects of AT1 receptor blockade by olmesartan, we used olmesartan at doses of 0.5 and 3 mg/kg per day, which did not affect systolic arterial pressure, heart rate, and plasma estrogen concentration (data not shown). Olmesartan at 3 mg/kg per day significantly decreased neointima formation 14 days after operation and decreased the number of BrdU-positive VSMCs in the media and neointima 7 days after operation in male, female, and OVX female mice, whereas these inhibitory effects of olmesartan were more marked in female mice (Figure 2). Olmesartan at a lower dose (0.5 mg/kg per day) did not affect neointima formation and BrdU index in male and OVX female mice, whereas this low dose of olmesartan significantly decreased neointima formation and BrdU index in intact female mice (Figure 2). Olmesartan and 17β-estradiol at lower doses (0.5 mg/kg per day and 20 μg/kg per day, respectively) did not affect neointima formation and BrdU index in OVX mice, whereas coadministration of both olmesartan and 17β-estradiol at these doses significantly decreased neointima formation and BrdU index (Figure 3).

Effect of Olmesartan and Estrogen on ERK and STAT Activation in Injured Artery

To examine the signaling mechanism by which estrogen and olmesartan effectively inhibited VSMC proliferation and decreased neointima formation in the injured artery, we focused on ERK activity, because the ERK pathway, which is activated by the AT1 receptor and growth factors, is critical for cell proliferation, differentiation, and, in some cells, hypertrophy.18 We examined the effect of olmesartan or estrogen on phosphorylation of ERK in the injured artery 7 days after cuff placement, by Western blotting. As shown in Figure 4A, cuff-induced vascular injury increased phosphorylation of ERK in the injured artery, whereas this increase in ERK phosphorylation was smaller in intact female mice than in male mice (5.9±0.8-fold increase in intact female mice versus 9.4±1.0-fold increase in male mice; P<0.05). Olmesartan at a dose of 3 mg/kg per day inhibited phosphorylation of ERK in the injured artery in both male and intact female mice. However, this inhibitory effect of olmesartan was greater in intact female mice than in male mice (76.3±6% inhibition in female versus 43.6±2% inhibition in male mice; P<0.05).

Figure 2. Effects of olmesartan (Olm) treatment on neointima formation and DNA synthesis in injured artery in male, intact female, and OVX mice. Mice were treated with olmesartan (0.5 or 3 mg/kg per day) using an osmotic pump implanted intraperitoneally at the time of cuff placement surgery. A, Morphometric analysis of cuff-placed artery in each group with and without olmesartan, 14 days after operation. B, BrdU incorporation in neointima and media of cuff-placed femoral artery 7 days after operation. *P<0.05 vs OVX. Values are mean±SEM; n=5 to 7 for each group.

Figure 3. Effects of olmesartan and/or estrogen on neointima formation and BrdU incorporation in injured artery in OVX mice. 17β-Estradiol (E2; 20 μg/kg per day) and olmesartan (Olm; 0.5 mg/kg per day) were administered as described in the Methods section. A, Morphometric analysis of cuff-placed artery 14 days after operation. B, BrdU incorporation in neointima and media of cuff-placed artery 7 days after operation. *P<0.05 vs OVX. Values are mean±SEM; n=5 to 7 for each group.

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We observed a further increase in ERK phosphorylation in OVX mice, and olmesartan at a dose of 3 mg/kg per day or 17β-estradiol at a dose of 80 μg/kg per day significantly decreased ERK phosphorylation in the injured artery (Figure 4B). As shown in Figure 4C, lower doses of 17β-estradiol (20 μg/kg per day) or olmesartan (0.5 mg/kg per day) alone did not significantly inhibit ERK phosphorylation, whereas coadministration of 17β-estradiol and olmesartan at these doses significantly inhibited ERK phosphorylation. Total protein level of ERK did not differ in each experimental group.

We also examined the tyrosine-phosphorylation of STAT1 and STAT3 in the injured artery, and we observed that phosphorylation of STAT1 and STAT3 was increased (Figures 5 and 6). These increases were significantly smaller in intact female mice than in male or OVX mice. Olmesartan (3 mg/kg per day) or 17β-estradiol (80 μg/kg per day) attenuated the phosphorylation of STAT in the injured artery. Additionally, the inhibitory effects of olmesartan were stronger in intact female mice. Lower doses of olmesartan (0.5 mg/kg per day) or 17β-estradiol (20 μg/kg per day) did not influence STAT phosphorylation in OVX mice, whereas coadministration of olmesartan and 17β-estradiol at these doses attenuated the phosphorylation of STAT1 and STAT3. Total protein levels of STAT1 and STAT3 did not differ in each experimental group.

**Discussion**

Consistent with previous reports, we demonstrated that neointima formation and DNA synthesis in the injured artery were less marked in intact female mice compared with those in male or OVX mice, and that estrogen replacement prevented the increase in neointima formation and DNA synthesis. The inhibitory effects of olmesartan on neointima formation and DNA synthesis in the injured artery were stronger in intact female than in male or OVX mice. Moreover, we demonstrated that coadministration of olmesartan and 17β-estradiol, even at lower doses, synergistically inhibited neointima formation and DNA synthesis in OVX mice. These results suggest that estrogen contributes at least partly to the ARB-mediated improvement of vascular remodeling, and that signaling crosstalk of the AT1 receptor and estrogen is important in the pathogenesis of vascular disease. Moreover, it has been reported that an ARB, candesartan cilexetil, reduced blood pressure more effectively than did the ACE inhibitor enalapril or the diuretic hydrochlorothiazide in women with mild to moderate hypertension, suggesting that the antihypertensive effect of ARB might be partly owing to estrogen.

We postulated that estrogen might inhibit AT1 receptor-mediated growth-promoting signals in the injured artery, thereby exaggerating the effect of ARB. To explore this possibility, we focused on ERK. We have recently demonstrated that estrogen inhibits AT1 receptor-mediated ERK phosphorylation in OVX mice, and olmesartan at a dose of 3 mg/kg per day or 17β-estradiol at a dose of 80 μg/kg per day significantly decreased ERK phosphorylation in the injured artery (Figure 4B). As shown in Figure 4C, lower doses of 17β-estradiol (20 μg/kg per day) or olmesartan (0.5 mg/kg per day) alone did not significantly inhibit ERK phosphorylation, whereas coadministration of 17β-estradiol and olmesartan at these doses significantly inhibited ERK phosphorylation. Total protein level of ERK did not differ in each experimental group.

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activation and cell proliferation in cultured VSMCs. Consistent with this in vitro finding, activation of ERK in the injured artery was significantly smaller in intact female mice than in male or OVX mice. Moreover, we observed that olmesartan or 17β-estradiol inhibited activation of ERK in the injured artery in vivo, and that even a lower dose of olmesartan effectively inhibited activation of ERK in vivo with a lower dose of 17β-estradiol. In contrast to our observation, van Eickel et al observed that estrogen did not affect ERK phosphorylation in the hypertrophic heart of mice after transverse aortic constriction, although it blocked p38-mitogen-activated protein kinase and attenuated pressure-overload cardiac hypertrophy, suggesting that p38-mitogen-activated protein kinase is involved in the estrogen-induced antihypertrophic effect.

**Figure 5.** Effects of olmesartan (Olm) and/or estrogen (E2) on tyrosine-phosphorylation of STAT1 in injured artery. Tissue samples were prepared from cuff-placed (cuff) arteries 7 days after operation. The tissue lysate was subjected to immunoblotting for phospho-STAT1 and STAT1. Upper panels, Representative results obtained from 4 independent experiments with different sample pools. Lower panels, Densitometric measurement of STAT1. Values are expressed as mean±SEM. *P<0.05 or †P<0.01 vs without olmesartan.

**Figure 6.** Effects of olmesartan (Olm) and/or estrogen (E2) on tyrosine-phosphorylation of STAT3 in injured artery. Tissue samples were prepared from cuff-placed (cuff) arteries 7 days after operation. The tissue lysate was subjected to immunoblotting for phospho-STAT3 and STAT3. Upper panels, Representative results obtained from 4 independent experiments with different sample pools. Lower panels, Densitometric measurement of STAT3. Values are expressed as mean±SEM. *P<0.05 or †P<0.01 vs without olmesartan.
c-fos gene expression is a critical determinant of VSMC proliferation and is regulated by the net interaction with different transcriptional factors. Inactivation of ERK may also result in decreased production of serum response factor, and this may act in concert with the inactivation of STATs, a component of the nuclear sis-inducing factor–complex, thereby resulting in a decrease of c-fos transcription. STATs are now known to be activated by many different extracellular signaling proteins, including cytokines, growth factors such as epidermal growth factor and platelet derived growth factor, and Ang II via the AT1 receptor. We demonstrated that in response to AT1 receptor stimulation, phosphorylated STAT1 and STAT3 accumulated in the nuclei of VSMCs and became a component of the nuclear sis-inducing factor–complex, resulting in enhancement of c-fos promoter activity. We observed that tyrosine-phosphorylation of STAT1 and STAT3 was increased in the injured artery, whereas these increases were significantly smaller in intact female mice than in male or O VX mice. We demonstrated that olmesartan or 17β-estradiol inhibited the activation of STATs in the injured artery in vivo, and that even a lower dose of olmesartan effectively inhibited the activation of ERK in vivo with lower dose of 17β-estradiol. These results suggest that estrogen-mediated inactivation of STATs might contribute to enhancement of the inhibitory effect of olmesartan on vascular injury. Analysis of the detailed mechanism of the estrogen-mediated increase in tyrosine-dephosphorylation of STAT1 and STAT3 may provide further understanding of the inhibitory effects of estrogen on VSMC proliferation and atherosclerosis.

In addition, estrogen has been shown to improve vascular remodeling by stimulating the release of NO and prostaglandin from vascular endothelial cells, suppressing collagen and elastin synthesis and/or deposition, and reducing the adhesion of activated monocytes to endothelium. We demonstrated that the beneficial effect of ARB to improve vascular remodeling is caused by not only blockade of the AT1 receptor but also stimulation of the unmasked AT2 receptor by Ang II. Therefore, it is possible that the effect of ARB is linked to the stimulation of AT2 receptor–mediated signaling such as activation of protein tyrosine phosphatases, which is further potentiated by estrogen. The estrogen-induced reduction of Ang II production might also contribute to the vascular protective effect. These results suggest that other mechanisms might be involved in the estrogen-mediated inhibitory effect on vascular remodeling with ARB. These possibilities have to be addressed, and more detailed analysis of the crosstalk of estrogen and Ang II is needed for further understanding of the pathogenesis of vascular remodeling and atherosclerosis.

Perspectives

In the present study, we showed that estrogen and ARB synergistically improve vascular remodeling after arterial injury, accompanied by marked inhibition of ERK and STAT activity, providing new insights into the negative crosstalk between the actions of estrogen and Ang II in vivo. Our findings provide novel insights into the pathogenesis of vascular remodeling and atherosclerosis, and might initiate rational and new therapeutic concepts. These results support the notion that a combination of ARB and estrogen replacement might be a useful and effective therapy for the treatment of cardiovascular diseases associated with the menopause.

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