Estrogen Modulates $\text{AT}_1$ Receptor Gene Expression
In Vitro and In Vivo

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Background—The $\text{AT}_1$ receptor has been implicated in the pathogenesis of hypertension and atherosclerosis. Estrogen deficiency is also associated with cardiovascular diseases. Therefore, we examined the $\text{AT}_1$ receptor gene expression in ovariectomized rats with and without estrogen replacement therapy and the influence of estrogen on $\text{AT}_1$ receptor expression in cultured vascular smooth muscle cells.

Methods and Results—Rat aortic tissue was examined 5 weeks after ovariectomy. In one group, estrogen (1.7 mg estradiol) was administered during the 5-week period. Functional experiments assessed angiotensin II–induced contraction of aortic rings. $\text{AT}_1$ receptor mRNA levels were measured by quantitative polymerase chain reaction and Northern blotting. $\text{AT}_1$ receptor density was assessed by radioligand binding assays. These techniques were also applied in cultured vascular smooth muscle cells. The efficacy of angiotensin II on vasoconstriction was significantly increased in aortas from ovariectomized rats. As assessed by radioligand binding assays, $\text{AT}_1$ receptor density was increased to 160% without changes in receptor affinity during estrogen deficiency. $\text{AT}_1$ receptor mRNA levels were consistently increased to 187% in ovariectomized rats compared with sham-operated animals. Estrogen substitution therapy in ovariectomized rats reversed this $\text{AT}_1$ receptor overexpression. To explore the underlying mechanisms, the direct influence of estradiol on $\text{AT}_1$ receptor expression was investigated in VSMCs. Estradiol (1 $\mu$mol/L) led to a time-dependent downregulation of $\text{AT}_1$ receptor mRNA, with a maximum of 33.3% at 12 hours. There was a correlative decrease in $\text{AT}_1$ receptor density.

Conclusions—This novel observation of estrogen-induced downregulation of $\text{AT}_1$ receptor expression could explain the association of estrogen deficiency with hypertension and atherosclerosis, because activation of the $\text{AT}_1$ receptor plays a key role in the regulation of blood pressure, fluid homeostasis, and vascular cell growth. (Circulation. 1998;97:2197-2201.)

Key Words: angiotensin ■ hypertension ■ hormones ■ genes ■ muscle, smooth ■ atherosclerosis

The low incidence of vascular diseases in premenopausal women and the rapid increase of the risk of cardiovascular events after menopause as well as the beneficial effects of estrogen replacement therapy on cardiac and vascular morbidity have suggested an important role of estrogens in the pathogenesis of atherosclerosis.1-3 In addition to its effects on classic cardiovascular risk factors, eg, in the sense of a decrease of cholesterol plasma levels,4,5 estrogen has been recognized to directly influence vascular as well as myocardial cells. Indeed, VSMCs, myocytes, and cardiac fibroblasts have been shown to contain functional estrogen receptors.6-8 Moreover, there is increasing evidence that estrogen interferes with the RAS. The production of angiotensinogen is enhanced, whereas ACE levels are decreased, by estrogens.9 According to a recent report, plasma renin levels are also reduced during estrogen replacement therapy, but other reports suggested either an increase or no change of plasma renin levels on estrogen treatment.10-13 One of the major components of the RAS is the $\text{AT}_1$ receptor, which mediates most biological effects of Ang II, such as vasoconstriction, aldosterone release, sodium and water retention, and cellular growth.14 The expression level of the $\text{AT}_1$ receptor is subject to regulation and governs the activity of the entire RAS through upregulation or downregulation. The $\text{AT}_1$ receptor is regulated by, eg, lipoproteins, growth factors, and Ang II in vitro as well as in vivo, suggesting the important role of this receptor in the development of atherosclerosis.15-18 To explore a potential involvement of $\text{AT}_1$ receptor regulation in the estrogen-induced modulation of cardiovascular diseases, we investigated the effects of estrogen deficiency on vascular $\text{AT}_1$ receptor expression in ovariectomized rats and the direct effect of estradiol on $\text{AT}_1$ receptor expression in VSMCs.
Methods

Animals

Wistar-Kyoto rats were ovarioctomized or sham-operated 8 weeks after birth. Tissue samples were harvested 5 weeks after surgery. For treatment, 17-estradiol pellets (containing 1.7 mg estradiol each, 60-day release, Innovative Research) were subcutaneously administered with a 10-gauge trochar. Plasma estradiol and renin levels were measured by standardized procedures (Amersham kit).

Functional Experiments

Rats were killed by decapitation 4 weeks after operation. Then the chest was rapidly opened, and the descending thoracic aorta was removed. The aorta was placed in chilled Krebs-Henseleit buffer and cleaned of excessive adventitial tissue. Eight 2- to 5-mm ring segments of thoracic aorta were suspended in individual organ chambers. When a stable baseline tone was established, potassium chloride, norepinephrine, endothelin, and Ang II were added at the concentrations indicated, interrupted by washout periods.

Cell Culture

VSMCs were isolated from rat thoracic aorta (female Wistar-Kyoto, 6 to 10 weeks old) by enzymatic dispersion as previously described. Cells were grown in a 5% CO₂ atmosphere at 37°C in estrogen-free and phenol-free DMEM supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 1% nonessential amino acids (×100), and 10% estrogen-free FCS.

mRNA Isolation, Northern Analysis, Quantitative PCR

After the indicated treatments, cells or isolated aortas were lysed with RNA-clean (AGS) and processed according to the manufacturer’s protocol to obtain total cellular RNA. Aliquots (10 μg) were electrophoresed through formaldehyde agarose gels, transferred onto Hybond N membranes, and then hybridized for 15 hours at 42°C with a random-primed [³²P]dCTP-labeled rat AT₁ receptor cDNA probe as described previously in detail. For quantitative PCR, isolated RNA was analyzed with a deletion-mutated AT₁ receptor mRNA as internal standard. PCR was performed under the same conditions and with use of the same specific primers as described previously.

Radioligand Binding Assays

VSMCs and aortic tissue were homogenized and membranes were isolated as described elsewhere. Ang II receptors were investigated in saturation experiments using [¹²⁵I]-labeled Ang II as radiolabeled ligand. The AT₁ receptor antagonist DUP 753 (10 μmol/L) was used to determine nonspecific binding. The incubation was carried out at 24°C for 60 minutes. All experiments were performed in triplicate. The maximal density (B₅₀) and apparent affinity (Kᵣ) of binding sites were obtained from nonlinear regression analysis.

Statistical Analysis

Data are presented as mean±SEM. Statistical analysis was performed by the one-factor ANOVA test using the Scheffé procedure.

Results

Plasma estradiol concentrations were significantly lower in male (7.7±0.8 pg/mL; P<0.05) and female ovarioctomized (3.0±0.3 pg/mL; P<0.05) rats than in sham-operated female animals (49.6±11.9 pg/mL). Blood pressure levels were not significantly different between the tested groups (data not shown).

To examine the functional response of aortas isolated from sham-operated female rats, ovarioctomized rats, and male rats, aortic constriction experiments were performed in the presence of various agonists. Vasoconstriction induced by 60 mmol/L potassium chloride was not significantly different between groups (data not shown). Ang II caused a significantly stronger vasoconstriction in ovarioctomized rats versus sham-operated rats, which was comparable to that of male rats (Figure 1A). The maximal constriction was increased in female rats from 12.8±1.6% to 17.4±1.1% (P<0.05) of KCl-induced vasoconstriction in ovarioctomized female rats (18.3±1.6% in male rats). The EC₅₀ values were not significantly altered. Control experiments using 0.1 mmol/L to 10 μmol/L phenylephrine and 10 mmol/L endothelin showed no significant difference between groups for either agonist (Figure 1B and 1C). The following experiments were conducted to clarify whether this profound increase in Ang II–caused vasoconstriction was based on increased AT₁ receptor expression. Quantitative PCR techniques showed that AT₁ receptor mRNA steady-state levels were significantly enhanced in aortas isolated from ovarioctomized rats versus sham-operated rats. AT₁ receptor mRNA levels were comparable in male rats and ovarioctomized female rats (Figure 1D). GAPDH mRNA was measured similarly in all groups (data not shown). Consequently, AT₁ receptor density was significantly increased in ovarioctomized rats compared with sham-operated rats. B₅₀ values were enhanced to 6.3±0.4 fmol/mg protein during estrogen deficiency versus 4.0±0.5 fmol/mg protein in normal female animals. The receptor affinities were not significantly different (Kᵣ, 1.1 nmol/L [0.6 to 1.6 nmol/L] in ovarioctomized versus 1.1 nmol/L [0.2 to 1.9 nmol/L] in sham-operated rats).

Renin plasma levels were measured to assess a possible compensatory modulation of the circulating RAS in response to the marked AT₁ receptor overexpression. Indeed, renin concentrations were significantly lower in ovarioctomized female rats (18.5±0.7 ng/mL) than in sham-operated female rats (23.3±1.8 ng/mL; P<0.05). Control experiments were conducted in which ovarioctomized rats were substituted with exogenous estrogen. Figure 1E illustrates that aortic AT₁ receptor mRNA was downregulated to control levels in ovarioctomized female animals after estrogen treatment, suggesting a decisive role for estrogens in gene regulation of the vascular AT₁ receptor. GAPDH mRNA remained unchanged (data not shown).

Most if not all vascular AT₁ receptors are expressed in VSMCs, and AT₁ receptor–mediated growth and vasoconstriction are realized predominantly through this cell type. To gain further mechanistic insight into the in vivo AT₁ receptor regulation during estrogen deficiency, we investigated the effects of estradiol on VSMCs in culture. Control experiments showed that AT₁ receptor and GAPDH mRNA levels remain stable over the experimental period of 24 hours (Figure 2A). Estradiol (1 μmol/L) caused downregulation of AT₁ receptor mRNA levels, with a maximal effect of 33.3±11% after a 12-hour incubation (Figure 2B). Radioligand binding assays on cells treated for 12 hours with estradiol confirmed that, like the AT₁ receptor mRNA, the AT₁ receptor density was significantly

Selected Abbreviations and Acronyms

Ang II = angiotensin II
PCR = polymerase chain reaction
RAS = renin-angiotensin system
VSMC = vascular smooth muscle cell
downregulated from $B_{\text{max}}$ values of 1327.3±183.3 fmol/mg protein in controls to 776.9±49.5 fmol/mg protein in estradiol-treated cells (Figure 2C). The receptor affinity was not significantly different ($K_d$ 1.7 nmol/L [1.1 to 2.2 nmol/L] versus 2.7 nmol/L [1.2 to 4.2 nmol/L]).

**Discussion**

Estrogen deficiency leads to upregulation of vascular AT$_1$ receptor expression accompanied by an increased effect of Ang II on tension in isolated aortic rings. This is presumably based on a direct downregulating effect of estradiol on AT$_1$ receptor gene expression in VSMCs.

VSMCs play a central role in the pathogenesis of atherosclerosis. Both estrogen and AT$_1$ receptors are expressed in this cell type; therefore, estrogens and Ang II may influence the intracellular processes of VSMCs. The AT$_1$ receptor decisively controls the events involved in VSMC growth and vasoconstriction. Because this receptor is subjected to homologous and heterologous regulation, its expression level governs the efficacy of the entire RAS. It is therefore conceivable that modulation of

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**Figure 1.** Functional effects of Ang II and AT$_1$ receptor expression in ovariectomized rats. Force of contraction in response to increasing concentrations of Ang II (A), phenylephrine (B), and 10 nmol/L endothelin (C) in aortic rings isolated from female ovariectomized, female sham-operated, and male rats. Each point represents mean±SEM, n=10, *P<0.05. D, AT$_1$ receptor mRNA levels in aortas from female ovariectomized, female sham-operated, and male rats as assessed by quantitative PCR. AT$_1$ receptor mRNA steady-state levels are expressed as ratio between wild-type and internal standard AT$_1$ receptor. Each point represents mean±SEM, n=5, *P<0.05. E, AT$_1$ receptor mRNA levels in aortas from female ovariectomized rats with estrogen treatment as assessed by quantitative PCR. AT$_1$ receptor mRNA steady-state levels are expressed as ratio between wild-type and internal standard AT$_1$ receptor mRNA.
STUDY METHODS

EXPERIMENTAL DESIGN

METHODS

RESULTS

CONCLUSIONS

ACKNOWLEDGMENTS

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