Estrogen Modulates AT<sub>1</sub> Receptor Gene Expression
In Vitro and In Vivo

Georg Nickenig, MD; Anselm T. Bämmer, MD; Christian Grohè, MD; Stefan Kahlert, PhD; Kerstin Strehlow, MS; Stephan Rosenkranz, MD; Alexander Stäblein, MD; Frank Beckers, MS; Jos F.M. Smits, MD; Mat J.A.P. Daemen, MD; Hans Vetter, MD; Michael Böhm, MD

**Background**—The AT<sub>1</sub> receptor has been implicated in the pathogenesis of hypertension and atherosclerosis. Estrogen deficiency is also associated with cardiovascular diseases. Therefore, we examined the AT<sub>1</sub> receptor gene expression in ovariectomized rats with and without estrogen replacement therapy and the influence of estrogen on AT<sub>1</sub> 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. AT<sub>1</sub> receptor mRNA levels were measured by quantitative polymerase chain reaction and Northern blotting. AT<sub>1</sub> 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, AT<sub>1</sub> receptor density was increased to 160% without changes in receptor affinity during estrogen deficiency. AT<sub>1</sub> receptor mRNA levels were consistently increased to 187% in ovariectomized rats compared with sham-operated animals. Estrogen substitution therapy in ovariectomized rats reversed this AT<sub>1</sub> receptor overexpression. To explore the underlying mechanisms, the direct influence of estradiol on AT<sub>1</sub> receptor expression was investigated in VSMCs. Estradiol (1 μmol/L) led to a time-dependent downregulation of AT<sub>1</sub> receptor mRNA, with a maximum of 33.3% at 12 hours. There was a correlative decrease in AT<sub>1</sub> receptor density.

**Conclusions**—This novel observation of estrogen-induced downregulation of AT<sub>1</sub> receptor expression could explain the association of estrogen deficiency with hypertension and atherosclerosis, because activation of the AT<sub>1</sub> 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. In addition to its effects on classic cardiovascular risk factors, eg, in the sense of a decrease of cholesterol plasma levels, 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. Moreover, there is increasing evidence that estrogen interferes with the RAS. The production of angiotensinogen is enhanced, whereas ACE levels are decreased, by estradiol. 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. One of the major components of the RAS is the AT<sub>1</sub> receptor, which mediates most biological effects of Ang II, such as vasoconstriction, aldosterone release, sodium and water retention, and cellular growth. The expression level of the AT<sub>1</sub> receptor is subject to regulation and governs the activity of the entire RAS through upregulation or downregulation. The AT<sub>1</sub> 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. To explore a potential involvement of AT<sub>1</sub> receptor regulation in the estrogen-induced modulation of cardiovascular diseases, we investigated the effects of estrogen deficiency on vascular AT<sub>1</sub> receptor expression in ovariectomized rats and the direct effect of estradiol on AT<sub>1</sub> 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 estrogen 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.18 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 described previously.17 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 standard. PCR was performed under the same conditions and with use of the same specific primers as described previously.19

Radioligand Binding Assays
VSMCs and aortic tissue were homogenized and membranes were isolated as described elsewhere.18 Ang II receptors were investigated in saturation experiments using 125I-labeled Ang II as radiolabeled ligand. The AT1 receptor antagonist DUP 753 (10 nmol/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 (Bmax) and apparent affinity (Kd) 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 nmol/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 nmol/L to 10 μmol/L phenylephrine and 10 nmol/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. Bmax 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 (Kd 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.14 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
downregulated from $B_{\text{max}}$ values of $1327.3 \pm 183.3$ fmol/mg protein in controls to $776.9 \pm 49.5$ 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...
AT1 receptor expression may lead to acceleration of pathophysiological events involved in the development of vascular disease.

In addition, estrogens putatively participate in the atherosclerotic process suggested by the rapid increase of vascular events in women after menopause.1–3 Several mechanisms have been proposed that may initiate this atheroprotective effect of estrogens. Among others, beneficial influence on classic risk factors,4,5 scavenging of free radicals, and interference with the RAS have been reported. In this context, enhanced production of angiotensinogen10,11 and reduced levels of ACE12,23 have been observed, whereas the effect of estrogens on renin are the subject of ongoing controversy.10,13,23

In ovariectomized rats, estrogen deficiency causes vascular overexpression of the AT1 receptor. In this short-term model, the blood pressure is not elevated significantly, probably because of the compensatory decrease of circulating renin levels. Our findings suggest that estrogen directly modulates AT1 receptor expression in VSMCs.

Recent reports showed that the AT1 receptor is overexpressed during conditions that are known to be associated with increased incidence of hypertension and atherosclerosis. LDL and salt cause a significant upregulation of AT1 receptor gene expression, leading to an enhanced biological efficacy of Ang II. Hypercholesterolemia and increased salt load not only lead to an accelerated progression of cardiovascular diseases but also are associated with overexpression of AT1 receptors, indicating that this receptor regulation may indeed participate decisively in the development of hypertension and atherosclerosis. In analogy, the incidence of hypertension and atherosclerosis is increased in estrogen-deficient women, possibly because of increased vasoconstriction and cell growth via overexpressed AT1 receptors. Estrogen replacement therapy causes a decreased risk for cardiovascular diseases; AT1 receptors are consistently downregulated by estrogens. Therefore, it may be concluded that upregulation of AT1 receptors during estrogen deficiency and the premenopausal, physiological “downregulation” of AT1 receptors are involved in the estrogen-driven effects on onset and development of hypertension and atherosclerosis.

Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft. The technical assistance of Marc Wolff and Kerstin Löbbert is greatly appreciated.

References


Estrogen Modulates AT₁ Receptor Gene Expression In Vitro and In Vivo

Circulation. 1998;97:2197-2201
doi: 10.1161/01.CIR.97.22.2197
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1998 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/97/22/2197

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/