Endothelial Dysfunction and Oxidative Stress During Estrogen Deficiency in Spontaneously Hypertensive Rats

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Background—Postmenopausal estrogen deficiency is associated with an increased cardiovascular risk, hypertension, and oxidative stress. Angiotensin type 1 (AT₁) receptor regulation is involved in the pathogenesis of atherosclerosis. To characterize vascular function, oxidative stress, and AT₁ receptor regulation during estrogen deficiency, ovariectomized spontaneously hypertensive rats (SHR) were investigated in comparison with sham-operated animals and with ovariectomized rats receiving estrogen replacement therapy with 17β-estradiol.

Methods and Results—Arterial blood pressure was similar in all 3 groups investigated. Five weeks after ovariectomy, endothelial dysfunction in aortic rings was observed, which was reversed by estrogen replacement therapy. Estrogen deficiency led to an enhanced vasoconstriction by angiotensin II. Vascular superoxide production was significantly increased compared with that in sham-operated rats, as measured by lucigenin chemiluminescence assays. Estrogen substitution normalized the production of free radicals in the vessel wall. Vascular AT₁ receptor expression was significantly upregulated by estrogen deficiency, as shown by quantitative reverse transcription–polymerase chain reaction, whereas endothelial NO synthase mRNA expression and NO release were unchanged. Five-week treatment of the animals with the AT₁ receptor antagonist irbesartan prevented endothelial dysfunction in ovariectomized rats and normalized the vascular production of free radicals.

Conclusions—In SHR, estrogen deficiency leads to increased vascular free radical production and enhanced angiotensin II–induced vasoconstriction via increased vascular AT₁ receptor expression, resulting in endothelial dysfunction. Estrogen replacement therapy and AT₁ receptor antagonism prevent these pathological changes. Therefore, estrogen deficiency–induced AT₁ receptor overexpression and oxidative stress may play an important role in cardiovascular diseases associated with menopause. (Circulation. 2001;103:435-441.)

Key Words: angiotensin ■ atherosclerosis ■ hormones ■ endothelium
which could ultimately lead to endothelial dysfunction. To test this hypothesis, we investigated spontaneously hypertensive rats after ovariectomy with and without concomitant estrogen replacement therapy. The significance of \(\text{AT}_1\) receptor activation was substantiated by an additional treatment regimen with the \(\text{AT}_1\) receptor antagonist irbesartan.

**Methods**

**Materials**

Angiotensin II, lucigenin, Taq DNA polymerase, nucleotides, salts, and other chemicals were purchased from Sigma Chemical Co. Moloney murine leukemia virus reverse transcriptase was obtained from GIBCO-BRL. Nitroglycerin was purchased from Solvay. RNA clean was purchased from AGS. Irbesartan was a gift from Sanofi-Synthelabo (Berlin, Germany).

**Animals**

Female spontaneously hypertensive rats (SHR) were put on a standard chow and were ovariectomized or sham-operated (control group) 16 weeks after birth. For treatment, 17\(\beta\)-estradiol pellets (containing 1.7 mg estradiol each, 60-day release, Innovative Research) were administered subcutaneously with a 10-gauge trocar. Irbesartan treatment was started 2 weeks after ovariectomy at 50 mg/kg \(1\text{d}\) \^\text{1}\text{d}\ by adding the drug to the drinking water.\text{\textsuperscript{20}} The rats were killed by decapitation. Animal experiments were performed in accordance with the German animal protection law. Tissue samples were harvested 5 weeks (7 weeks for the irbesartan group) after surgery.

**Blood Pressure Measurement**

Animals were anesthetized (100 mg/kg body wt IP ketamine and 5 mg/kg body wt IP xylazine), and a stretched PE catheter was inserted into the femoral artery and exteriorized at the neck. The animals were allowed to recover from anesthesia for 48 hours before the blood pressure measurements were performed by connecting the saline-filled catheter to a pressure transducer. Measurements took place in conscious animals 5 times for 10 minutes each on 2 consecutive days. Thereafter, the animals were anesthetized as described above and killed by decapitation, and the organs were explanted.

**Aortic Ring Preparations and Tension Recording**

After excision of the descending aorta, the vessel was immersed in chilled modified Tyrode’s buffer (\(pH 7.4\)) composed of (mmol/L) NaCl 136.9, KCl 5.4, CaCl\(_2\) 1.8, MgCl\(_2\) 1.05, Na-EDTA 0.05, Na\(_2\)HPO\(_4\) 0.42, NaHCO\(_3\) 22.6, and \(d\text{+}\)glucose 5.5, which contained additional ascorbic acid (0.28 mmol/L) and indomethacin (0.01 mmol/L). Adventitial tissue was carefully removed. Five-millimeter rings were mounted for recording of isometric tension in organ baths filled with modified Tyrode’s buffer (37°C), which was continuously aerated with 95% O\(_2\)/5% CO\(_2\). The preparations were attached to a force transducer, and isometric tension was recorded on a polygraph. Aortic rings were allowed to equilibrate for 60 minutes. A resting tension of 1 g was maintained throughout the experiment. Drugs were added in increasing concentrations to obtain cumulative concentration-response curves: KCl (20 and 60 mmol/L), angiotensin II (0.01 mmol/L to 1 \(\mu\)mol/L), phenylephrine (0.1 mmol/L to 10 \(\mu\)mol/L), carbachol (0.1 mmol/L to 100 \(\mu\)mol/L), and nitroglycerin (1 nmol/L to 10 \(\mu\)mol/L). The drug concentration was increased when vasoconstriction or vasorelaxation was completed (on average, 3 to 6 minutes for each step). Drugs were washed out before the next substance was added.

**mRNA Isolation and PCRs**

Aortas were isolated, quickly frozen in liquid nitrogen, and homogenized with a motorized homogenizer. RNA was isolated with RNA clean according to the manufacturer’s protocol to obtain total cellular RNA. Aliquots (1 \(\mu\)g) were electrophoresed through 1.2% agarose–

0.67% formaldehyde gels and stained with ethidium bromide to verify the quantity and quality of the RNA. Isolated total RNA (1 \(\mu\)g) and an \(\text{AT}_1\) receptor mutant mRNA (10 pg) were mixed and reverse-transcribed by using random primers and Moloney murine leukemia virus reverse transcriptase for 60 minutes at 42°C and 10 minutes at 75°C. The single-stranded cDNA was amplified by polymerase chain reactions (PCRs) by using Taq DNA polymerase. Twenty-eight cycles were performed under the following conditions: 30 seconds at 94°C, 45 seconds at 55°C, and 45 seconds at 72°C. The sequence for \(\text{AT}_1\) receptor sense and antisense primers were 5’-ACCCCTCTACGCATCATCTTTGTTGTOGGG-3’ and 5’-GGGCGTGTCGAAATTCCGGGACCTAAATGTA-3’; respectively. The same cDNA samples were used for GAPDH cDNA amplification (22 cycles) to confirm that equal amounts of RNA were reverse-transcribed. The primers used were 5’-ACCCAGTGCCATGCATAC-3’ and 5’-TCCACACACCTGTTGCTGTA-3’. PCR amplification gave 479-, 191-, and 452-bp fragments that originated from \(\text{AT}_1\) receptor wild-type mRNA, mutated \(\text{AT}_1\) receptor mRNA, and GAPDH mRNA, respectively. Amplification of a 340-bp fragment of \(\text{eNOS}\) cDNA was carried out with primer pairs 5’-TCCGCGTCCACCTGATCCTA-3’ and 5’-AACATATGTCCCTTGCTCAAGCA-3’ for 35 cycles under the following conditions: 30 seconds at 94°C, 30 seconds at 60°C, and 60 seconds at 72°C. For semiquantification, PCR conditions were chosen so that the reaction was within the linear exponential phase with respect to the amount of cDNA template and number of cycles performed. Equal amounts of reverse transcription (RT)-PCR products were loaded on 1.5% agarose gels, and optical densities of ethidium bromide–stained DNA bands were quantified. \(\text{AT}_1\) receptor mRNA expression was expressed as the ratio of \(\text{AT}_1\) receptor wild-type and \(\text{AT}_1\) receptor mutant (internal standard) PCR signal of each sample.

**Measurement of ROS**

For measurement of superoxide release of intact vessel segments, aortas were excised carefully and placed in chilled modified Krebs-HEPES buffer (\(pH 7.4\)) composed of (mmol/L) NaCl 99.0, KCl 1.87, MgSO\(_4\) 1.20, Na-HEPES 20.0, K\(_2\)HPO\(_4\) 1.03, NaHCO\(_3\) 25.0, and \(d\text{+}\)glucose 11.1. Connective tissue was removed, and aortas were cut into 5-mm segments. The aortic rings were placed in Krebs-HEPES buffer aerated with 95% O\(_2\)/5% CO\(_2\) and were incubated for 30 minutes at 37°C. Then the samples were transferred into scintillation vials containing 2 mL Krebs-HEPES buffer with 5 \(\mu\)mol/L lucigenin. Chemiluminescence was assessed over 10 minutes in a scintillation counter (Berthold Lumat LB 9501) at 1-minute intervals. Background signals were subtracted. The vessel segments were then dried, and dry weight was determined. Superoxide release is expressed as relative chemiluminescence per milligram aortic tissue.

**NO Measurement**

Excised and prepared aortic segments were placed in oxygenated (\(P_O2\) 150 mm Hg) 10 mmol/L HEPES buffer. The vessel was longitudinally opened and placed in an organ bath with the luminal face turned upward. An NO-sensitive electrode (ISO-NO electrode, World Precision Instruments) was placed at a fixed distance of 1 mm above the aortic lumen. Beforehand, the electrode was calibrated with a standardized NO solution. Substances were added at the same place in the organ bath, and NO release of the aortic segment was measured.

**Statistical Analysis**

Data are presented as mean±SEM obtained in at least 3 separate experiments. Statistical analysis was performed by ANOVA (post hoc Scheffé procedure) and Mann-Whitney U test with SSPS 6.0 software. A value of \(P<0.05\) indicates statistical significance.

**Results**

**Estrogen Plasma Concentrations**

Estrogen plasma levels dropped in ovariectomized rats (1.6±0.5 pg/mL) compared with sham-operated rats (35.7±12 pg/mL) and recovered after estrogen substitution (61±21 pg/mL).
Effect of Estrogen Deficiency on Blood Pressure in SHR

Blood pressure was evaluated intra-arterially in conscious animals. Blood pressure levels (4 weeks after ovariectomy) were not significantly different between groups: systolic blood pressures were 160±4 mm Hg for sham-operated rats, 170±12 mm Hg for ovariectomized rats, and 178±9 mm Hg for ovariectomized rats with estrogen replacement (n=5 per group).

Effect of Estrogen Deficiency on Aortic Vasorelaxation and Vasoconstriction

Aortic rings were isolated 5 weeks after ovariectomy, and their functional performance was assessed in organ chamber experiments (n=5 with 15 rings per group). Figure 1 shows the endothelium-dependent vasorelaxation on increasing concentrations of carbachol and the endothelium-independent relaxation exerted by nitroglycerin. Whereas the endothelial cell–independent vasorelaxation was not altered by ovariectomy, the carbachol-induced vasodilation was impaired during estrogen deficiency, suggesting a decremental effect of estrogen deficiency on endothelial function in SHR. Nitroglycerin-induced vasodilatation at concentrations of 10 nmol/L and 1 μmol/L nitroglycerin was impaired during estrogen replacement therapy (P<0.05 versus control). Endothelial function was improved after estrogen replacement therapy of ovariectomized rats, which supports the notion that estrogen selectively influences endothelial function. Nitroglycerin-induced vasodilatation at concentrations of 10 nmol/L and 1 μmol/L nitroglycerin was impaired during estrogen replacement therapy (P<0.05 versus control). However, ED₅₀ values and maximal efficacy remained unaltered.

The contraction of the aortas was assessed during exposure to increasing concentrations of either phenylephrine or angiotensin II. Figure 2 reveals that the angiotensin II–induced vasoconstriction was selectively increased after ovariectomy (force of contraction 2.0±0.1 mN for control; angiotensin II, 0.1 μmol/L; P<0.05 versus control). This hypercontractility on angiotensin II stimulation was completely abolished by estrogen replacement treatment. In
contrast, α-adrenoreceptor–mediated vasoconstriction induced by phenylephrine was not altered significantly.

Effect of Estrogen Deficiency on Vascular Superoxide Production

The increased vascular responsiveness on angiotensin II in ovariectomized SHR could possibly lead not only to enhanced vasoconstriction but also to an enhanced level of free radicals in the vessel wall, which could cause the observed endothelial dysfunction. Therefore, the vascular production of ROS was assessed by lucigenin chemiluminescence assays in intact isolated aortic segments (n = 10 per group). Figure 3 illustrates that estrogen deficiency induced a significant increase of superoxide production in the vessel wall to 160 ± 27% of control levels (P < 0.05 versus control), which was completely prevented by concomitant estrogen replacement therapy (P < 0.05 versus ovariectomy).

Effect of AT1 Receptor Blockade on Endothelial Function and Superoxide Release During Estrogen Deficiency

The above-mentioned findings suggest that enhanced AT1 receptor activation causes endothelial dysfunction as well as enhanced oxidative stress. To further support this notion, ovariectomized SHR were treated with the AT1 receptor antagonist irbesartan for 5 weeks. Vasomotion of aortic ring preparations was assessed in organ chamber experiments (n = 5 with 15 rings per group). Figure 1 reveals that AT1 receptor antagonism completely normalized endothelial dysfunction in estrogen-deficient rats (P < 0.05 versus ovariectomy). Nitroglycerin-induced vasorelaxation was similar between the groups. Endothelial function in either sham-operated or estrogen-treated animals was not altered (data not shown).

Endothelial function is likely to be improved by the reduction of oxidative stress. Figure 3 demonstrates that the treatment with irbesartan significantly decreased vascular superoxide production in ovariectomized SHR (P < 0.05 versus ovariectomy).

Effect of Estrogen Deficiency on Vascular AT1 Receptor and eNOS mRNA Expression

Estrogen deficiency of SHR caused an increase of angiotensin II–induced vasoconstriction and vascular ROS production. Both effects are prominently mediated through AT1 receptor activation. Therefore, it was reasonable to assume that estrogens directly influenced vascular AT1 receptor expression. Vascular AT1 receptor mRNA concentrations were assessed by means of quantitative RT-PCR in RNA isolated from aortic segments of all SHR groups. Figure 4A shows the densitometric analysis (n = 5 per group), revealing that AT1 receptor mRNA expression was significantly up-
regulated to 177±26% of control in ovariectomized SHR (P<0.05 versus control). Treatment of ovariectomized rats with estrogens reversed this AT1 receptor overexpression (P<0.05 versus ovariectomy). Figure 4B demonstrates the unaltered GAPDH expression (n=5 per group). In addition, eNOS mRNA expression was assessed in the same samples via semiquantitative RT-PCR. Figure 4C illustrates the densitometric results of these experiments (n=5 per group). Expression of eNOS mRNA remained unchanged between groups.

**Effect of Estrogen Deficiency on Vascular NO Release**

Estrogen-induced increase of vascular NO release could also account for the worsening of endothelial dysfunction during estrogen deficiency. Therefore, the NO release of aortic segments was selectively measured with an NO electrode. Figure 5 shows that carbachol-induced NO release was not statistically different between groups (n=7 per group), suggesting that estrogen-induced modulation of NO release or production was not involved in the detected alterations of vascular function.

**Discussion**

Estrogens have been suggested to exert vasoprotective effects, leading to a considerable lowering of cardiovascular events.1–3 This notion is based on several epidemiological, clinical, and molecular findings: The incidence of cardiovascular disease is low in premenopausal women, but it increases steadily in postmenopausal women. Additionally, postmenopausal hormone replacement therapy may reduce this rise of cardiovascular events, as suggested by retrospective studies.3–5 Earlier reports attributed the beneficial vascular effects of estrogens mainly to their influence on serum lipid concentrations.3 Recently, evidence is accumulating that direct effects of estrogens on blood vessels may contribute significantly to their cardioprotective effects.19 This involves long-term effects on cellular gene expression programs, which are thought to be mediated by genomic effects of the activated steroid receptors. In addition, non-genomic rapid effects in vascular cells, which obviously occur independently of modulation of gene expression, have been reported. One of the most prominent features seems to be a rapid vasodilatory effect of estrogen, which is elicited through endothelium-dependent and endothelium-independent mechanisms. Current data suggest that estrogens enhance the bioavailability of NO through stimulation of eNOS (NOS III) and NO release and potentially also through antioxidant properties.10–12,21

These effects are of special interest with respect to the pathogenesis of atherosclerosis. Namely, increased release of NO has been associated with improved endothelial dysfunction and inhibition of cell growth. Endothelial dysfunction is not only a prerequisite of atherosclerosis but seems to serve also as a potent predictor of cardiac event rates.22–25 Besides NO, ROS are thought to be involved in the onset and development of endothelial dysfunction. Endothelial cells and vascular smooth muscle cells are known to be potent sources of ROS.15,17,18,26 It has recently been shown that these molecules participate in the proliferation of vascular smooth muscle cells, promote the development of hypertension, and influence the apoptosis of vascular cells,17,26–28 which may be related to either oxidative scavenging of NO or to direct cellular effects of free radicals.29 Recent findings suggest that an overwhelming production of ROS, such as superoxide and hydrogen peroxide, rather than a decreased production of NO may be decisively involved in the initiation and the acceleration of vascular damage.16

AT1 receptor activation induces vasoconstriction and cellular growth and leads to free radical release in the vessel wall.29 This receptor is highly regulated, among others, by angiotensin II, lipoproteins, growth factors, and insulin.30–33 It has recently been reported that estrogen causes downregulation of the vascular AT1 receptor and that estrogen deficiency is accompanied by AT1 receptor overexpression.19 On the basis of these findings, we reasoned that estrogen deficiency could lead to increased oxidative stress and endothelial dysfunction via AT1 receptor regulation.

Indeed, the present study indicates that estrogen deficiency causes endothelial dysfunction in SHR, which is presumably mediated through increased oxidative stress, as assessed by the enhanced superoxide production in the vessel wall. Expression of eNOS and NO release were not altered by ovariectomy or estrogen replacement therapy, suggesting that not a decrease in NO synthesis but rather an enhanced production of free radicals such as superoxide underlies the observed endothelial dysfunction. The latter may be evoked by AT1 receptor overexpression during estrogen deficiency, which was reversed by estrogen therapy. The prevented AT1 receptor overexpression during estrogen supplementation led to decreased oxidative stress and to an improved endothelial function.

The presented data suggest that vascular eNOS expression and NO release are not influenced by estrogens in this model; these findings seem to be contradictory to the aforementioned findings on estrogen-induced NO release.10–12,21 Whereas our
results are derived from a long-term animal model, data on estrogen-evoked NO release are mostly derived from short-term in vitro studies, which may explain the contrasting findings. Moreover, it has been reported that estrogens did not enhance eNOS expression and activity in mouse and rat models or in cultured endothelial cells, which supports our presented data.

To further explore the role of AT₁ receptor activation in the setting of estrogen deficiency, ovariectomized rats were concomitantly treated with an AT₁ receptor antagonist. This treatment not only normalized vascular superoxide production but also reversed the endothelial dysfunction associated with estrogen deficiency without replacement of estrogens. This strongly suggests that the detected AT₁ receptor overexpression in the absence of estrogens may play a decisive role in the enhanced vascular damage after ovariectomy. This is also documented by the fact that angiotensin II caused a profoundly increased vasoconstriction in the ovariectomized animals. According to our data, the antioxidant properties of estrogens could at least in part be mediated through the downregulation of AT₁ receptor gene expression.

Our findings are in good agreement with a recently published study that showed, in comparison with an antihypertensive regimen, a more potent reduction of blood pressure by AT₁ receptor antagonism in postmenopausal women. Especially in the light of the Heart and Estrogen/Progestin Replacement Study (HERS) trial, a prospective secondary prevention study that did not show a beneficial influence of estrogen replacement therapy on cardiovascular mortality in postmenopausal women, alternative treatment strategies for women at coronary risk after menopause need to be evaluated. AT₁ receptor overexpression in the pathophysiological setting of estrogen deficiency and the profound antihypertensive effect of AT₁ receptor antagonists provide new mechanistic insights and medical tools that could help to introduce a more successful prevention of cardiovascular events in postmenopausal females.

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References


