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

Females suffer less frequently from cardiovascular diseases during their reproductive years than do their male counterparts. This tendency disappears after menopause, the natural state of estrogen deficiency.1,2 Estrogen replacement therapy potentially prevents the development of cardiovascular diseases in postmenopausal women.3–5 The vascular effects of estrogens are not completely understood. Estrogens lower plasma lipoproteins,3 influence the renin-angiotensin system,6,7 exert antioxidative properties,8 and may act as calcium-blocking agents.9 In addition, estrogens exert direct effects on the vessel wall, such as an increase of vascular NO production and modulation of endothelial NO synthase (eNOS [NOS III]) expression.10–12

Increased NO production and the modulation of the lipid profile may in part underlie the well-recognized beneficial effects of estrogens on endothelial dysfunction, a prerequisite of atherosclerosis.13,14 However, it is currently thought that endothelial dysfunction is not based on reduced production but is evoked by a decreased bioavailability of NO.15,16 The latter is decisively influenced by the level of reactive oxygen species (ROS), such as superoxide, in the vessel wall. An increased production of superoxide putatively leads to the scavenging of NO and to the cellular damage associated with endothelial dysfunction.15,16

Angiotensin type 1 (AT1) receptor activation is a predominant source of free radical production in the vasculature.17,18 Recently, it has been shown that estrogen deficiency causes AT1 receptor overexpression in vivo, leading to enhanced biological effects of the renin-angiotensin system that could in part serve as an explanation for the increase in cardiac events after menopause in women.19

We hypothesized that a lack of estrogens could induce increased oxidative stress via AT1 receptor overexpression,
which could ultimately lead to endothelial dysfunction. To test this hypothesis, we investigated spontaneously hypertensive rats after ovariec-
yomy with and without concomitant estrogen replacement therapy. The significance of AT1 receptor activation was substantiated by an additional treatment regimen with the AT1 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. 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 ovariec tomized or sham-operated (control group) 16 weeks after birth. For treatment, 17β-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 ovariecotomy at 0.5 mg/kg/day by adding the drug to the drinking water.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 99.01, KCl 11.8, MgCl2 1.05, Na2EDTA 0.05, NaH2PO4 0.42, NaHCO3 22.6, and d(+)-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% O2/5% CO2. 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% O2/5% CO2. 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 μmol/L), phenylephrine (0.1 μmol/L to 10 μmol/L), carbachol (0.1 mmol/L to 100 μmol/L), and nitroglycerin (1 mmol/L to 10 μmol/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 μg) 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 μg) and an AT1 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 AT1 receptor sense and antisense primers were 5'-ACCACTTCAGCATCATTCTGTTGTOGGG-3' and 5'-GGGACGTCGAATCCAGAGCATAATGGA-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'-ACACACGTTCCATCCAATC-3' and 5'-TCCACACCTGTTGCTGA-3'. PCR amplification gave 479, 191, and 452-bp fragments that originated from AT1 receptor wild-type mRNA, mutated AT1 receptor mRNA, and GAPDH mRNA, respectively. Amplification of a 340-bp fragment of eNOS cDNA was carried out with primer pairs 5'-TTCCGCTGCCACCTGATCCTAA-3' and 5'-AACATAGTGTCCCCAGCAAGCA-3' for 35 cycles under the following conditions: 20 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. AT1 receptor mRNA expression is expressed as the ratio of AT1 receptor wild-type and AT1 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 99.01, KCl 4.69, CaCl2 1.87, MgSO4 1.20, Na2-HEPES 20.0, K2HPO4 1.03, NaHCO3 25.0, and d(+)-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% O2/5% CO2 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 μmol/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 (P02 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 ovariec tomized 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 ± 6 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 (force of contraction 18.6 ± 4.8% versus 3.4 ± 1.0% for control of phenylephrine-induced vasoconstriction; carbachol, 100 μmol/L; 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, ED_{50} 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 versus 1.4±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
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-

![Figure 3](image-url)  
**Figure 3.** Effect of estrogen deficiency and AT1 receptor blockade on vascular production of ROS. Superoxide production in intact isolated aortic segments was assessed by lucigenin chemiluminescence assays (5 μmol/L lucigenin). Superoxide release is expressed as relative chemiluminescence (RLU) per milligram of aortic tissue (mean ± SEM, n = 7 to 10 per group). *P < 0.05 vs sham; **P < 0.05 vs ovariectomized SHR (Ovarex).

![Figure 4](image-url)  
**Figure 4.** Effect of estrogen deficiency on vascular AT1 receptor and eNOS expression. AT1 receptor mRNA expression in aortic preparations of sham-operated control rats and estrogen-deficient SHR was assessed by quantitative RT-PCR; expression of eNOS and GAPDH mRNA was quantified by semiquantitative RT-PCR. Densitometric analysis was expressed as mean ± SEM (n = 5 per group) of ratio of AT1 receptor wild-type (AT1-R WT) and AT1 receptor mutant (AT1-R mutant) PCR signal (A). Densitometric analysis of amplified GAPDH (B) and eNOS DNA fragments (C) is expressed as mean ± SEM (n = 5 per group). *P < 0.05 vs sham; **P < 0.05 vs ovariectomized SHR (Ovarex).
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 cardiac events.21 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.1–3 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.21 This involves long-term effects on cellular gene expression programs, which are thought to be medi-

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.18 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 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 AT1 receptor gene expression in vitro and in vivo. The effects of estrogen 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 women.

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