Improvement of Endothelial Dysfunction by Selective Estrogen Receptor-α Stimulation in Ovariectomized SHR


Abstract—Both known estrogen receptors, ERα and ERβ, are expressed in blood vessels. To gain further insight into the role of ERα in a functional setting, we investigated the effect of the novel highly selective ERα agonist Cpd1471 on vascular reactivity in ovariectomized spontaneously hypertensive rats (SHR). After ovariectomy or sham operation, 12-week-old female SHR received either 17β-estradiol (E2, 2 μg/kg body wt per day), the selective ERα agonist Cpd1471 (30 μg/kg body wt per day), or placebo. Acetylcholine-induced endothelium-dependent vasorelaxation was significantly blunted in aortas from ovariectomized rats (R_{max}, 53%±3% versus sham, 79%±2%; P<0.001). Treatment with E2 or Cpd1471 significantly augmented acetylcholine-induced relaxation in ovariectomized rats (R_{max}, 70%±2%; resp, 73%±2%). Endothelium-independent relaxation induced by sodium nitroprusside was not different among the four groups. The contractile response induced by the nitric oxide (NO) synthase inhibitor Nω-nitro-L-arginine, an index of basal NO formation, was significantly lower in ovariectomized rats compared with sham-operated animals (53±2% versus 77%±5%; P<0.01) and was normalized by both E2 (70%±2%) and Cpd1471 (70%±3%). Aortic endothelial NO synthase (eNOS) expression and phosphorylation of the vasodilator-stimulated phosphoprotein, an index of NO/cGMP-signaling, was reduced in ovariectomized SHR and normalized by E2 and Cpd1471. In SHR after ovariectomy, endothelium-dependent NO-mediated vasorelaxation and eNOS expression are attenuated. The novel selective ERα agonist Cpd1471 prevented these pathophysiological changes to a similar extent as E2. Thus, the pharmacological principle of selective ERα activation mediates positive vascular effects. (Hypertension. 2003;42:991-996.)

Key Words: estrogen | endothelium | nitric oxide | nitric oxide synthase | rats, spontaneously hypertensive

Gender differences in the risk for cardiovascular diseases are well recognized, with premenopausal women exhibiting a lower risk than age-matched men. The advantage of women over men in cardiovascular morbidity disappears after menopause, suggesting that estrogen plays an important role in cardiovascular health.1 Estrogens are known to exert beneficial effects on the vascular wall. Long-term estrogen treatment improves endothelial dysfunction, a major contributor to the pathophysiology of cardiovascular disease, through upregulation of endothelial cell genes, such as endothelial nitric oxide synthase (eNOS).2-4 Furthermore, estrogen has rapid nongenomic effects on the vascular endothelium, including activation of nitric oxide (NO) synthesis.5,6 However, despite the positive effects on vascular function in animal models7-9 and humans,10-14 estrogen replacement therapy with 17β-estradiol or mixtures of equine estrogens as in the Heart and Estrogen/progestin Replacement Study (HERS) has failed to protect from cardiovascular diseases in large controlled clinical trials.15-18 Therefore, in recent years, research has focused on selective estrogen receptor modulation as a possible new pharmacological principle.19

Estrogen effects occurring at physiological hormone concentrations are mediated by estrogen receptors, which are transcription factors belonging to the family of steroid hormone receptors. Both estrogen receptor subtypes known, ERα and ERβ, are encoded by different genes and are expressed in vascular endothelial and smooth muscle cells.20,21 Positive cardiovascular effects and especially the increase in NO production by estrogen have been reported to involve ERα22-24 as well as ERβ.25,26

To bring the differential effects of ERα and ERβ closer to the clinical situation, we investigated for the first time the influence of a novel highly selective ERα agonist on vasorelaxation and eNOS expression in isolated aortas from spontaneously hypertensive rats (SHR) after ovariectomy. In addition, we measured the phosphorylation status of the vasodilator-stimulated phosphoprotein (VASP) to determine
the functionality of the NO/cGMP-mediated vasodilatory pathway in vivo.27

Methods

Study Protocol, Hemodynamic Measurements
Twelve-week-old female SHR (Charles River) were ovariectomized or sham-operated under isoflurane anesthesia. Starting 1 day after ovariectomy, animals were randomly selected for daily subcutaneous injection of 17β-estradiol (E2, 2 μg/kg body wt per day), the selective ERα agonist Cpd1471 (30 μg/kg body wt per day), or placebo. All animals had free access to standard rat chow and water. E2 and Cpd1471 were dissolved in ethanol and injected with peanut oil used as a carrier substance; placebo animals received ethanol/peanut oil alone. After 4 weeks of treatment, blood pressure was recorded as described previously28 and animals were euthanized. Estradiol serum levels were measured by radioimmunoassay (DPC-Biernann).

Pharmacological Properties of Cpd1471
The binding affinity of Cpd1471 to ERα and ERβ was determined by competition experiments, using cytosol preparations from rat uterus (containing ERα) or rat prostate (containing ERβ). [3H]-17β-estradiol served as the radioactive ligand; unlabeled 17β-estradiol was used as the reference. The relative binding affinity of Cpd1471 to ERα proved to be 125-fold higher compared with the relative binding affinity to ERβ. Transactivation assays with Cpd1471 were performed with U2-OS human osteosarcoma cells transfected with either ERα or ERβ and an estrogen-sensitive reporter gene (ERE-ERE-luciferase).29 Cpd1471 exhibited 200-fold higher relative potency (compared with the reference 17β-estradiol) in cells transfected with ERα compared with ERβ.

Vascular Reactivity Studies
The descending thoracic aorta was dissected and carefully cleaned of connective tissue. A 10-mm segment was immediately frozen in liquid nitrogen for Western blot analysis. Another segment was cut into rings (3 mm in length), which were mounted in an organ bath (Führ Medical Instruments) for isometric force measurements. The rings were equilibrated for 30 minutes under a resting tension of 2 g in oxygenated (95% O2; 5% CO2) Krebs-Henseleit solution (NaCl 118 mmol/L, KCl 4.7 mmol/L, MgSO4 1.2 mmol/L, CaCl2 1.6 mmol/L, KH2PO4 1.2 mmol/L, NaHCO3 25 mmol/L, glucose 12 mmol/L; pH 7.4, 37°C) containing diclofenac (1 μmol/L) to exclude influences of cyclooxygenase products. Rings were repeated contracted by KCl (100 mmol/L) until reproducible responses were obtained. Thereafter, the relaxant response to cumulative doses of acetylcholine after preconstriction with 50 mmol/L KCl was assessed. Contractile response of aortic rings induced by KCl was not different among the four groups of SHR.

To evaluate the formation of basal NO, the contraction induced by 45 minutes of incubation with the NO-synthase inhibitor Nω-nitro-L-arginine (L-NA, 100 μmol/L) was measured in ring segments preconstricted with phenylephrine to ~10% of maximum contraction. Endothelium-independent relaxation was assessed with the use of sodium nitroprusside.

Western Blot Analysis
Aortic tissue was homogenized in ice-cold RIPA buffer. These crude protein extracts were subjected to SDS–PAGE electrophoresis and transferred to nitrocellulose membranes. Proteins were detected by using their specific antibodies and visualized by enhanced chemiluminescence (eNOS) or with the Odyssey Infrared Imaging System (LI-COR) (VASP).

A more detailed description of the Western blot analysis can be found in an online supplement available at http://www.hypertensionaha.org.

Materials
All biochemistries were obtained in the highest purity available from Sigma. The selective ERα agonist Cpd1471 was kindly provided by Schering, AG (Berlin, Germany).

Statistics
Relaxant responses were given as percentage relaxation relative to the preconstriction level. Values are expressed as mean±SEM of n experiments with segments from different animals. Statistical analysis was performed by 1-way ANOVA for repeated measurements, followed by a post hoc Bonferroni test. Probability values <0.05 were considered statistically significant.

Results

Global Parameters
Mean arterial blood pressure did not differ between ovariectomized and sham-operated SHR (193±5 resp, 188±4 mm Hg), but it tended to be slightly lower in ovariectomized rats supplemented with either E2 or Cpd1471 (173±5 resp, 171±7 mm Hg; NS). Uterus weight, a long-term parameter of ERα activation, was lower in ovariectomized placebo-treated compared with sham-operated rats (91.8±12.1 resp, 288.1±16.5 mg; P<0.001) and increased substantially by treatment with E2 (173.0±17.8 mg) and Cpd1471 (160.5±17.9 mg). Body weight was increased in ovariectomized placebo-treated compared with sham-operated rats (244±2 resp, 199±3 g; P<0.001) and lowered by treatment with E2 (212±4 g) and Cpd1471 (225±3 g). Estradiol serum levels were significantly lower in ovariectomized SHR treated with placebo or Cpd1471 (24.7±0.9 resp, 28.4±1.8 pg/mL; P<0.01) compared with sham-operated animals (74.8±31.7 pg/mL) and normalized by E2 treatment (103.6±10.4 pg/mL).

Vascular Reactivity in Aortic Rings
In preconstricted aortic rings, acetylcholine induced a concentration-dependent relaxation that was blunted in aortas from ovariectomized rats (Figure 1). The concentration–relaxation curves to the endothelium-independent relaxant sodium nitroprusside were not different between sham-operated and ovariectomized rats (Figure 2). Treatment with either the selective ERα agonist Cpd1471 or E2 significantly augmented the acetylcholine-induced relaxation of aortic rings from ovariectomized rats (Figure 1) without affecting the response to sodium nitroprusside (Figure 2).

The NO synthase inhibitor L-NA induced a significantly higher contraction in sham-operated than in ovariectomized animals (Figure 3), indicating a reduced basal NO formation in rings from ovariectomized SHR. L-NA–induced contraction in aortic rings from ovariectomized SHR was significantly augmented either by Cpd1471 or E2, suggesting a higher NO formation in these segments.

Expression of Endothelial NO Synthase in Rat Aorta
eNOS protein expression was decreased in ovariectomized rats as compared with sham-operated rats (Figure 4). Treatment with either the selective ERα agonist Cpd1471 or E2 normalized eNOS protein expression in aortas from ovariectomized rats.
Phosphorylation of VASP Serine\textsuperscript{239} in Rat Aorta

VASP phosphorylation at serine 239, a biochemical marker of the functionality of cyclic nucleotide–mediated vasodilatory pathways in the vessel wall,\textsuperscript{27} was reduced in aortas from ovariectomized SHR compared with sham-operated animals. Treatment with the selective ER\textalpha agonist Cpd1471 (E2, +) enhanced acetylcholine-induced relaxation in aortic rings from ovariectomized rats (Figure 5). Total VASP expression was similar among the four groups.

**Discussion**

In the present study, we demonstrated for the first time that a subtype-selective ER\textalpha agonist prevents endothelial dysfunction and downregulation of eNOS protein expression in ovariectomized SHR to the same extent as non–subtype-selective ER\textalpha and ER\beta stimulation by 17\beta-estradiol.
Estrogen has an important role in the maintenance of cardiovascular health in women. After menopause, the natural state of estrogen deficiency, women are at higher risk for cardiovascular diseases. Estrogen is a potent vasoprotective molecule exerting indirect and direct effects on cardiovascular tissues. The indirect effects such as lowering of plasma lipoprotein levels and modulating the fibrinolytic system account for approximately one third of the beneficial cardiovascular effects. Direct actions of estrogen on the vascular wall, including changes in endothelial cell gene expression and function, substantially contribute to its cardioprotective effects. Estrogens exert their biological effects on vascular cells by rapid nongenomic and classic genomic mechanisms: rapid activation of eNOS induces acute vasodilation; upregulation of eNOS expression confers long-term benefit. As confirmed in the present study in SHR, estrogen deficiency induces endothelial dysfunction in animal models as well as in humans, which can be restored by estrogen supplementation. Observational studies have shown that women without documented cardiovascular disease taking hormone replacement therapy have a reduced risk of major cardiovascular events compared with untreated women. However, large controlled clinical trials, such as HERS, failed to show a beneficial effect on secondary prevention in women with heart disease and even suggested an increased risk of cardiovascular events. In the HERS trial, hormone replacement therapy was associated with a significantly increased risk of cardiovascular events within the first year and thromboembolic events (deep venous thrombosis and pulmonary embolism). Several mechanisms may account for the disappointing results in these end point trials, for example, concomitant use of medroxyprogesterone may mask positive effects of conjugated estrogens, and the study population in the HERS trial was significantly older than the average postmenopausal women prescribed hormone replacement therapy. However, there is no clear explanation for the negative results in these clinical end point studies. With the development of selective estrogen receptor modulators acting as estrogen receptor antagonist in the breast and uterus and as estrogen receptor agonist in the vasculature, such as raloxifene, the hope was raised to induce only the positive effects and exclude the negative side effects of the nonselective estrogen receptor stimulator E2 such as deep venous thrombosis and pulmonary embolism. Indeed, studies in animals as well as in humans revealed that selective estrogen receptor modulation can mediate positive cardiovascular effects, such as improving endothelium-dependent vasodilation.

Besides selective receptor modulation, selective activation of ERα and ERβ, both of which differ in tissue-specific expression and biological function, appears as a novel pharmacological principle to improve the safety and efficiency of estrogens in cardiovascular disease. To gain further insight into the role of ERα in a functional setting and to identify novel compounds for clinical application, we investigated the influence of a novel and highly selective ERα agonist on endothelial function in SHR. The essential new finding of the present study is the preservation of endothelium-dependent vasorelaxation after ovariectomy in SHR by a chronic treatment with the novel subtype ERα selective agonist Cpd1471. The SHR is a well-known and widely used animal model of hypertension with documented aggravation of endothelial dysfunction after ovariectomy compared with sham-operated animals. Therefore, ovariectomized SHR constitute a suitable model to study estrogen deficiency and the effect of replacement therapy. Indeed, after ovariectomy, endothelium-dependent vasorelaxation was significantly reduced in placebo-treated SHR. Furthermore, we found a diminished contraction induced by the NO synthase inhibitor L-NA in aortic rings from ovariectomized placebo-treated rats, indicating a reduced basal NO formation in these rats. To further elucidate the functionality of NO/cGMP signaling in the aorta, we measured VASP phosphorylation at serine by using a phosphorylation-specific antibody. NO induces vasorelaxation by stimulating the soluble guanylyl cyclase, increasing the level of cGMP and activating cGMP-dependent protein kinases type I (cGK-I). VASP, a protein localized at actin filaments, focal adhesions, and dynamic membrane regions, is a validated substrate of cGK-I. Hence, phosphorylation of VASP at serine is a suitable biochemical marker for cGK-I activity and has been shown to reflect NO bioavailability in the vascular wall under physiological as
well as pathophysiological conditions. Estrogen deficiency after ovariectomy resulted in attenuation of VASP phosphorylation in the aortas of SHR that could be restored by either E2 or the selective ERα agonist Cpd1471. Taken together, our data suggest that the reduced protein expression of eNOS in estrogen-deficient SHR diminishes NO/cGMP signaling in the vessel wall, leading to blunted endothelium-dependent relaxation. Although rapid nongenomic effects were not the focus of the present study, the demonstration that long-term E2 therapy increased eNOS protein expression in aorta from SHR is in line with previous observations obtained in cultured endothelial cells.2–4

The relative importance of ERα and ERβ in cardiovascular disease is still a matter of debate, although the genomic effects of estrogen have been attributed mainly to stimulation of ERα by estrogen. To date, it was unclear whether a subtype ERα-selective agonist would be as effective in inducing beneficial vascular effects as E2. Biochemical studies demonstrated a 125-fold-higher binding affinity of Cpd1471 for ERα than for ERβ. In transactivation assays, Cpd1471 exhibited 200-fold-higher relative potency if acting through ERα compared with ERβ. Prevention of endothelial dysfunction by Cpd1471 in SHR after ovariectomy in the present study shows that selective ERα stimulation is sufficient to mediate beneficial vascular effects. Indeed, Cpd1471 increased eNOS protein expression and VASP phosphorylation to the same extent as E2.

Perspectives
It is established that estrogen has beneficial cardiovascular effects. However, there is a striking discrepancy between animal and human studies showing positive effects on vascular wall morphology and function and large controlled clinical end point trials demonstrating increased cardiovascular events by hormone replacement therapy. The role of the two estrogen receptors, ERα and ERβ, which may mediate positive cardiovascular effects, to date was only investigated by cell culture studies or by knockout models. Using an in vivo model of estrogen deficiency, we for the first time demonstrated that chronic treatment with a novel highly selective ERα agonist mediates positive vascular effects. Although we do not rule out that ERβ effects also contribute to E2-mediated vasoprotection, our data suggest that pharmacological ERα stimulation alone is sufficient enough to prevent endothelial dysfunction elicited by estrogen deficiency. Further studies will be necessary to investigate whether ERα stimulation is superior to E2 treatment regarding negative side effects of hormone replacement therapy such as thrombotic events. However, the pharmacological principle of subtype-specific estrogen receptor stimulation gives the hope for a new treatment option for cardiovascular protection.

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