Prolonged Ovarian Hormone Deprivation Impairs the Protective Vascular Actions of Estrogen Receptor α Agonists

Christian Pinna, Andrea Cignarella, Paola Sanvito, Valeria Pelosi, Chiara Bolego

Abstract—The vascular consequences of estrogen treatment may be driven by its initiation timing. We tested the hypothesis that the duration of ovarian hormone deprivation before estrogen reintroduction affects the role of estrogen as mediator of endothelial function and vascular relaxation in nondiseased vessels. Rats were ovariectomized and implanted with 17β-estradiol (E₂) or oil capsules 1, 4, and 8 months after surgery. After the longest hypoestrogenicity period, acetylcholine-mediated aortic relaxation was attenuated and insensitive to E₂ administration despite endothelial integrity. Whereas no rapid vasorelaxant responses were elicited by an estrogen receptor (ER) β-selective agonist, responses to E₂ and an ERα selective agonist waned postovariectomy at any given time and were restored by E₂ treatment after 1 and 4 months but not 8 months postovariectomy. Accordingly, endothelial ERα mRNA and protein expression declined ≈6-fold after prolonged hypoestrogenicity and was restored by estrogen replacement starting 1 month but not 8 months postovariectomy. Furthermore, the amount of active phosphorylated endothelial NO synthase rose significantly after E₂ replacement after 1 and 4 months but not 8 months postovariectomy. The present findings document that the functional impairment of the ERα/endothelial NO synthase signaling network after an extended period of hypoestrogenicity was not restored by E₂ administration, providing experimental support to early initiation of estrogen replacement with preferential ERα targeting to improve cardiovascular outcomes. (Hypertension. 2008; 51:1210-1217.)

Key Words: endothelium ■ hormones ■ pharmacology ■ NO synthase ■ receptors

In spite of a large body of preclinical studies attesting beneficial actions of estrogenic treatment on the cardiovascular system, large clinical trials of hormone therapy so far have failed to improve clinical outcomes (reviewed in Reference 1). In attempting to explain the apparent discrepancy between experimental and clinical results, the timing of treatment initiation has been deemed a critical factor. The timing hypothesis proposes that the earlier an estrogenic treatment starts, the more likely it is of being successful, because the time since menopause is a major risk factor for the development and progression of atherosclerosis.2,3 This is consistent with observations that cyclic or permanent changes in circulating concentrations of estrogen in premenopausal and postmenopausal women, respectively, affect vascular responses.4,5 Of note, estrogen deprivation in rats time-dependently impairs endothelial function, as assessed by the loss of acetylcholine-mediated dilation, but this response is restored by early 17β-estradiol (E₂) replacement.6 In addition, estrogen affects endothelial vasomotor responses per se. We demonstrated previously that ovariectomy abolishes acute estrogen dilation, which is restored by timely E₂ replacement.7 Thus, vascular relaxation is a primary target of estrogen action in the vessel wall. This is known to occur through rapid stimulation of NO production and modulation of NO synthase genes.5,8,9

The cellular responses to estrogens are mediated by interactions with either nuclear- or membrane-located estrogen receptor (ER)α or ERβ,10 of which the expression in the vasculature is highly regulated both by endocrine status and pathological conditions. For instance, ERα gene expression decreases after ovariectomy in rat cerebral arteries and increases after E₂ replacement therapy11 without changes in ERβ gene expression.12 The 2 ER isoforms not only are differentially modulated by circulating hormones but also mediate distinct actions in the vascular wall. In fact, ERα conveys both short-term effects including endothelial dilation and long-term anti-inflammatory actions of E₂.13 In this regard, we demonstrated previously that ERα-selective agonists, unlike ERβ-selective agonists, induce acute vascular relaxation in the aorta from intact female or E₂-replaced ovariectomized (OVX) rats.7

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Endothelial Isolation

Thus, the present study was designed to investigate whether incremental periods of estrogen deprivation affect rapid functional vasomotor responses to estrogenic agents and/or long-term effects of E₂ treatment. In particular, we examined the impact of timing of E₂ treatment initiation after ovariec
tomy on endothelial function by measuring the following: (1) rapid ex vivo aortic vasorelaxation induced by nonselective (E₂) and novel selective ERα agonists; (2) accumulation of the eNOS protein and its active phosphorylated form (peNOS); and (3) expression of ERα.

Methods

Drugs and Reagents

Noradrenaline (NA), acetylcholine chloride, and E₂ were purchased from Sigma-Aldrich. Propylpyrazole triol (PPT) and diapropyli
nitrile (DPN) was purchased from Tocris Cookson Inc. NA and acetylcholine were dissolved in distilled water, whereas E₂ and PPT were freshly dissolved in ethanol at a concentration of 1 mmol/L; further dilutions were obtained in Krebs’ solution. The final ethanol concentration in organ baths did not exceed 0.01%.

Animal Protocol

Experiments were performed on isolated aortic rings excised from female Sprague-Dawley rats initially weighing 200 to 225 g (Charles River, Calco, Italy). The animals were kept in temperature-controlled facilities on a 12-hour light/dark cycle and fed normal chow. Bilateral ovariectomy was performed under ketamine (40 mg/kg IP) and xylocaine (20 mg/kg IP) anesthesia. OVX animals were divided into 3 groups and euthanized after 1, 4, and 8 months since ovarian ablation. Each group received subcutaneous implant of 2 silastic capsules containing 25 µL of vehicle (peanut oil) or E₂ (235 µg/µL; 0.86 mmol/L) for 5 days before sacrifice.16,17 Plasma E₂ concentrations after treatment approached the normal rat proestrus level.16 All of the procedures were performed in accordance with the guidelines for laboratory animal care of Milan University.

Isolated Organ Bath Experiments

The aorta was carefully removed, cleaned of fat and connective tissue, and cut into 5- to 6-mm rings. Vessels were suspended in 5-mL organ baths containing Krebs’ solution at 37°C, continuously bubbled with 95% O₂ and 5% CO₂. The Krebs’ solution had the following composition: 118 mmol/L of NaCl, 4.7 mmol/L of KCl, 1.2 mmol/L of KH₂PO₄, 1.1 mmol/L of MgSO₄, 2.5 mmol/L of CaCl₂, 25 mmol/L of NaHCO₃, and 5.5 mmol/L of glucose (pH 7.4). The rings were connected to isometric tension transducers (FT 10, WP1) coupled with a digital recording system (PowerLab 8SP, ADInstruments). Vascular tissues were equilibrated for 30 minutes and contracted with 0.1 µmol/L of NA to develop a maximal response. Preparations were then washed with fresh Krebs’ solution, and the equilibration period was allowed to continue for an additional 30 minutes. Experiments were carried out on tissues precontracted with NA to 60% of maximal contraction. Cumulative concentration-response curves for E₂ and PPT (ERα selective agonist) were obtained over the concentration range 0.01 pmol/L to 100 nmol/L. To test vehicle effects, cumulative additions of equivalent ethanol dilutions were also performed. Cumulative concentration-response curves were also obtained for acetylcholine (10⁻⁷ to 10⁻⁵ mol/L). Relaxant re-
sponses were expressed as the percentage of relaxation of NA-precontracted tissues.

Histology and Immunohistochemistry

The thoracic aorta was longitudinally opened on a Petri dish. The endothelial layer was removed by gently scraping the lumen and transferred into a 1.5-mL Eppendorf tube containing PBS. The tissue pooled from 2 aortas was centrifuged at 8000 rpm for 5 minutes and the supernatant discarded. The pellet was dissolved in RNA lysis solution and frozen at −80°C until RNA analysis or resuspended in 30 µL of lysis buffer18 and frozen until protein analysis. Immunodetection of the endothelial marker eNOS in 15 µg of lysate protein gave similar results to that performed in 15 µg of protein from cultured rat aorta and human umbilical vein endothelial cells.7

Western Blot Analysis

After 4 freeze-thaw cycles, endothelial protein lysates were boiled for 5 minutes and centrifuged at 13 000 rpm for 15 minutes. Total protein was determined using Lowry’s method. Cell lysate protein was size fractionated on 10% SDS-polyacrylamide gels. After electrophoresis, proteins were transferred onto Hybond ECL membranes (GE HealthCare). After blocking with 5% nonfat dry milk (BioRad), membranes were hybridized overnight with anti-eNOS (1:800, Santa Cruz Biotechnology), anti-peNOS (pS1177, phospho-specific, BD Transduction Laboratories), or anti-ERα antibody (1:500, Santa Cruz Biotechnology); washed; and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature. After extensive washing, membranes were developed using enhanced chemiluminescence reagents (GE HealthCare). The uniformity of protein load and transfer efficiency across the test samples was verified with Ponceau staining.
Aortic Rings From OVX Rats Implanted 1, 4, or 8 Months After Surgery With Oil (n
obtaining concentration-response curves to acetylcholine, an
tested using arterial rings isolated from thoracic aorta by
animals restored uterine weight at all of the time points,
P\(0.05\) were considered significant.

Quantitative Real-Time PCR
RNA was extracted using a commercially available kit (Ambion). RNA (1 µg) was reverse transcribed using random hexamer primers and SuperScript (Invitrogen). Amplification was performed on the Taqman 7000 (Applied Biosystems) using FastStart TaqMan Probe Master (Roche Applied Sciences), gene-specific TaqMan probes (Applied Biosystems) and primers, and a standard 2-steps thermal cycler protocol (95°C for 15 seconds and 60°C for 1 minute, repeated 40 times). Relative quantification of gene expression was calculated by the comparative C\(_T\) method and normalized to the eukaryote housekeeping gene 18S.

Statistical Analysis
All of the data are expressed as means±SEMs of 4 to 5 independent experiments, each value representing means±SEMs of duplicate or triplicate determinations. Concentration-response curves were obtained with software Prism (GraphPad Software Inc). Potency (pD\(_2\)) values and maximal responses (E\(_{\text{max}}\)) to pharmacological agents were compared by 1-way ANOVA followed by Bonferroni’s posthoc test as appropriate, using Minitab software. Western blot and real-time PCR data are the means±SEMs of 3 independent experiments. The comparison between groups (OVX versus E\(_2\)-treated rats) was performed by 1-way ANOVA. Values of \(P<0.05\) were considered significant.

Results
The hormone regimen used in this study produces circulating E\(_2\) concentrations of 52 to 58 pg/mL.\(^{16}\) E\(_2\) treatment in OVX animals restored uterine weight at all of the time points, whereas it had no effect on body weight (Table 1).

The functional capacity of the endothelium ex vivo was tested using arterial rings isolated from thoracic aorta by obtaining concentration-response curves to acetylcholine, an endogenous endothelium-dependent vasodilator triggering eNOS-mediated NO production. Acetylcholine induced full vascular relaxation with comparable efficacy and potency in precontracted aortic tissues from oil- and E\(_2\)-treated rats after 1 and 4 months of hypoestrogenicity (Figure 1A and 1B). By contrast, acetylcholine-induced relaxation was impaired in aortic tissues from 8-month OVX oil-treated rats and was not improved by E\(_2\) replacement (Figure 1C). The pharmacological parameters of acetylcholine efficacy (E\(_{\text{max}}\)) and pD\(_2\) in treatment groups are shown in Table 2. After endothelial function assessment, rapid responses to increasing concentrations of exogenous E\(_2\) (0.01 pmol/L to 100 nmol/L) were recorded in aortic preparations. No vasodilating response was induced by E\(_2\) in tissues from oil-replaced rats irrespective of the duration of estrogen deprivation (Figure 2A through 2C). A significant 22% relaxation to the exogenous hormone was observed in tissues from E\(_2\)-replaced rats that underwent surgery 1 and 4 months earlier (Figure 2A and 2B). By contrast, no significant relaxant responses (\(P>0.05\) versus oil) were elicited by exogenous E\(_2\) in aortic tissues from 8-month OVX E\(_2\)-replaced rats (Figure 2C). The pharmacological parameters of E\(_2\) efficacy (E\(_{\text{max}}\)) and pD\(_2\) in treatment groups are shown in Table 2. Because the rapid vascular relaxation to E\(_2\) is mediated solely by ER\(\alpha\),\(^{7}\) we also obtained concentration-response curves to the ER\(\alpha\) selective agonist PPT. The relaxant responses to this agent were entirely overlapping to those elicited by the physiological nonselective ER ligand E\(_2\) (Figure 2A through 2C). The pharmacological parameters of

Table 2. Pharmacologic Assessment of E\(_{\text{max}}\) and pD\(_2\) (log EC\(_{50}\)) From Concentration-Response Curves to Acetylcholine, E\(_2\), and PPT in Aortic Rings From OVX Rats Implanted 1, 4, or 8 Months After Surgery With Oil (n=4) or E\(_2\) (n=5) Capsules for 5 Days

<table>
<thead>
<tr>
<th>Months</th>
<th>Ach</th>
<th>E(_2)</th>
<th>PPT</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Oil</td>
<td>E(_{\text{max}})</td>
<td>pD(_2)</td>
</tr>
<tr>
<td>1</td>
<td>7.6±0.1</td>
<td>94.5±5.5</td>
<td>7.4±0.2</td>
</tr>
<tr>
<td>4</td>
<td>7.4±0.1</td>
<td>100±0.0</td>
<td>7.3±0.1</td>
</tr>
<tr>
<td>8</td>
<td>7.3±0.1</td>
<td>73.4±0.1†</td>
<td>7.1±0.2</td>
</tr>
</tbody>
</table>

NA indicates not applicable; Ach, acetylcholine.

\(^{*}\)P<0.05 compared with oil.

†P<0.05 compared with 1 month.
PPT efficacy (Emax) and pD2 in treatment groups are shown in Table 2. To confirm that ERα/H9252 is not involved in rapid vasorelaxation, we obtained concentration-response curves to the ERα selective agonist diarylpropionitrile, which failed to affect vascular tone irrespective of OVX duration and E2 replacement (Figure 3A through 3C). The pharmacological parameter of diarylpropionitrile efficacy (Emax) in treatment groups is shown in Table 3.

To test whether morphological changes in the vasculature after 8-month ovariectomy rendered the vessel nonresponsive to E2-induced relaxation, sections of the aorta were obtained at different time points. Hematoxylin-eosin staining (Figure 4) provided no evidence for visible injury or gross vascular changes irrespective of OVX duration and E2 replacement, suggesting the occurrence of functional rather than morphological impairment of the endothelium after long-term ovariectomy.

Because eNOS is a key determinant of endothelium-dependent relaxation, as well as an effector of estrogen action in the vessel wall, we assessed the amount of the active peNOS in endothelial lysates from rats at different time since ovarian surgery. The effectiveness of E2 treatment was witnessed by the 3- to 4-fold increase in peNOS versus oil in the endothelium from rats that were deprived of estrogen for 1 or 4 months (Figure 5A) as shown by Western blotting experiments. By contrast, the amount of endothelial peNOS did not increase after E2 treatment in the endothelium from rats that had been deprived of estrogen for 8 months (Figure 5A). Total eNOS protein levels in the aortic endothelium of oil-treated rats were also increased by E2 treatment after 1 or 4 months postovariectomy (Figure 5B), yet to a lower extent as compared with peNOS. As shown with peNOS above, E2 reintroduction after 8 months led to nonsignificant changes in eNOS protein levels (Figure 5B).

In attempting to define a potentially unifying explanation for the functional and biochemical events in the arterial wall described so far, we found that E2 treatment doubled endothelial ERα mRNA expression in tissues from 1-month OVX rats as assessed by real-time PCR (Figure 6). Although a similar relative increase in ERα mRNA levels was observed in the endothelium of 8-month OVX E2-treated rats, ERα expression dropped dramatically in 8 months as compared with 1-month OVX rats (Figure 6). ERα protein as measured by Western blotting was upregulated by E2 treatment in keeping with real-time PCR data (Figure 7) and was identical to a standard recombinant...
ERα protein (Figure 7). Finally, immunohistochemical analysis for ERα, p-eNOS, and eNOS in aortic tissues from 1-month as compared with 8-month OVX E2-treated animals revealed marked negative regulation of the former proteins with less apparent changes in the levels of the more abundantly expressed eNOS (Figure 8). This suggests that prolonged hypoestrogenicity disrupted the ERα/eNOS signaling network in nondiseased rat arteries.

Discussion
To the best of our knowledge, this is the first demonstration that E2 treatment did not exert beneficial effects on the biology of the arterial wall in rats after prolonged estrogen deprivation, such as that ensuing after 8 months postovariectomy, while retaining efficacy when administered within a window of 4 months after ovariectomy. Although the vascular actions of E2 are abrogated in surgically postmenopausal atherosclerotic monkeys,19 the present study provides possible mechanisms whereby the vascular actions of exogenous E2 in nondiseased vessels were abrogated simply on prolonged deprivation of the endogenous hormone. In this context, 2 key players were identified in dictating vascular estrogen efficacy, namely, ERα and eNOS. The 2 proteins are organized into a functional signaling module in caveolae as demonstrated in cultured endothelial cells.15,20 Although it is well established that eNOS is modulated by E2selective agonists through both genomic and nongenomic activation of ERα,23,24 the present findings render ERα and eNOS as primary targets for the loss of efficacy of delayed E2 reintroduction after ovarian hormone deprivation. In addition, the present findings are relevant to rapid actions of ER agonists, as well as long-term effects of E2 replacement against the background of estrogen deprivation. The fact that ERα agonists, at least in part, induce their protective effects via NO, as first shown by Rosselli et al,25 suggest that NO or endothelial-dependent relaxation may serve as an important marker to assess whether estrogen therapy should be initiated or not.

Acetylcholine is often used to determine the functional capacity of the endothelium. In postmenopausal women, intracoronary infusion of estrogen selectively enhances endothelium-dependent dilation by acetylcholine, supporting a role for estrogen in acetylcholine-mediated responses.26 In the present study, E2 reintroduction after 8 months postovariectomy failed to enhance attenuated acetylcholine-mediated dilation because of prolonged E2 deprivation, differently from what observed previously in 3- to 18-month OVX rats with estrogen replacement starting on the day of surgery.6 Furthermore, E2 reintroduction after 8 months but not 1 or 4 months postovariectomy failed to restore the rapid relaxant response to nonselective and ERα-selective agonists that was abolished by estrogen deprivation (Figure 2 and Table 2). Because endothelial integrity was not affected by long-term ovariectomy (Figure 4), these results emphasize that uncontrolled long-term ovariectomy impaired endothelial function so that it became insensitive to estrogen replacement.

Our results also indicate that a major pathway of E2’s beneficial actions in the arterial wall was enhanced eNOS activation through phosphorylation of Ser1177, which is mediated by the phosphatidylinositol 3-kinase/Akt pathway in endothelial cells.20,27 Accordingly, the increase in eNOS and even more so in p-eNOS levels in response to E2 replacement in 1- and 4-month OVX animals was blunted in 8-month OVX animals most likely because of the marked fall in ERα expression observed in the same tissues, resulting in functional impairment of the ERα/eNOS signaling network. Consistent with the functional assessment using nonselective and ERα-selective agonists, ERα mRNA and protein expression sharply decreased after long-term as compared with short-term ovariectomy and was positively regulated by E2 treatment regardless of timing. This is in contrast to the previous observation of nonsignificant changes in ERα expression as evaluated (not quantitatively) in the thoracic aorta (not in the endothelium) after OVX and E2 treatment,28 although the schedule of ovariectomy and treatment with E2 in that study largely differed from ours. Our data also illustrate that the positive regulation of ERα mRNA and protein levels by E2 treatment after 8 months postovariectomy restored only ~20% of the ERα mRNA levels seen in the

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<th>Months</th>
<th>Oil</th>
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<tr>
<td>1</td>
<td>pD2</td>
<td>E_max</td>
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<tr>
<td></td>
<td>NA</td>
<td>91.2±0.1 NA</td>
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<td>4</td>
<td>NA</td>
<td>96.5±1.5 NA</td>
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<td>8</td>
<td>NA</td>
<td>94.8±1.5 NA</td>
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NA indicates not applicable.
endothelium of oil-treated 1-month OVX rats (Figures 6 and 8), making it unlikely that such a reduced ERα pool was capable of mediating biological actions of E2 therapy. Thus, loss of ERs in the artery wall occurs not only in atherosclerotic arteries but also in nondiseased vessels on prolonged estrogen deprivation. Although ERβ expression was not assessed in the present study, this ER isoform does not appear to be relevant to rapid vasorelaxant responses to ER agonists (Figure 3 and Reference 7). On the other hand, the efficacy of our E2 replacement regimen was documented by the uterotrophic effect independent of hypoestrogenicity duration. Overall, prolonged hypoestrogenicity induced genomic effects on ERα expression that had profound impacts on its signaling network and vascular biology in terms of outcome of E2 administration and rapid endothelium-dependent relaxation to estrogenic agents. It should be pointed out, however, that additional ER-dependent and independent mechanisms, including inhibitory effects on smooth muscle cell growth, may account for protective effects of E2 on the vasculature.

Perspectives
This study provides experimental proof of concept that the timing of initiation of E2 treatment after loss of ovarian...
hormone production is critical to therapeutic cardiovascular outcomes. Accordingly, recent animal studies show that prolonged hypoestrogenicity suppresses the neuroprotective and anti-inflammatory actions of \( \text{E}_2 \). Beyond basic science contributions, the timing issue was raised by observational studies and validated to some extent by secondary analysis of the large-scale Women’s Health Initiative Trial, with further trials ongoing. Thus, the time may have come to integrate findings from fundamental research into clinical practice to successfully exploit the beneficial actions of estrogenic agents on the cardiovascular system.

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**Disclosures**

None.

**References**


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