Estrogen Causes Dynamic Alterations in Endothelial Estrogen Receptor Expression

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Abstract—Estrogen receptor (ER)α mediates many of the effects of estrogen on the vascular endothelium. The purpose of the present study was to determine whether estrogen modifies endothelial ERα expression. In experiments in cultured ovine endothelial cells, physiological concentrations of 17β-estradiol (E2, 10^-10 to 10^-8 mol/L) caused an increase in ERα protein abundance that was evident after 6 hours of hormone exposure. Shorter (2-hour) E2 treatment caused ERα downregulation. In contrast to the upregulation in ERα after long-term E2, the expression of the other ER isoform, ERβ, was downregulated. Both nonselective ER antagonism with ICI 182,780 and the inhibition of gene transcription with actinomycin D blocked the increase in ERα with E2. In studies using the human ERα gene promoter P-1 coupled to luciferase, an increase in ERα gene transcription was evident in endothelial cells within 4 hours of E2 exposure. The transcriptional activation was fully blocked by ICI 182,780, whereas the specific ERβ antagonist RR-tetrahydrochrysene yielded partial blockade. Overexpression of ERα or ERβ caused comparable 10- and 8-fold increases, respectively, in ERα promoter activation by E2. Thus, long-term exposure to E2 upregulates ERα expression in endothelial cells through the actions of either ERα or ERβ on ERα gene transcription; in contrast, E2 causes ERβ downregulation in the endothelium. We postulate that E2-induced changes in ERα and ERβ expression modify the effects of the hormone on vascular endothelium. (Circ Res. 2002;91:814-820.)

Key Words: endothelium ■ estrogen ■ estrogen receptor α ■ estrogen receptor β

There is considerable evidence from studies in both human and animal models that estrogen plays an important role in cardiovascular health.1 Whereas approximately one third of the clinical advantages of estrogen can be attributed to changes in circulating lipid levels,2-5 there are also important direct effects on the vascular wall, including changes in endothelial cell gene expression and function. Long-term estrogen exposure upregulates the expression of endothelial cell genes, including cyclooxygenase (COX)-1 and endothelial NO synthase (eNOS),6-8 and it inhibits cytokine-mediated endothelial cell adhesion molecule expression.9 Estrogen also has rapid nongenomic effects on endothelial cells, which include NO activation and prostacyclin production within minutes of hormone exposure.10-13 It is most likely a combination of genomic and nongenomic processes that underlies the capacity of estrogen to have a beneficial impact on vascular health.1

The actions of estrogen in most paradigms are mediated by estrogen receptors (ERs), which classically serve as transcription factors. There are two known ER subtypes, designated ERα and ERβ, and the expression of both isoforms has been observed in endothelial cells in multiple model systems.14 The two ER isoforms share significant homology in their DNA binding and ligand binding domains (95% and 55%, respectively), but they vary greatly in their N terminal (A/B) domains, which contain the ligand-independent transcriptional activation function TAF-1. ERα and ERβ also have opposite effects on the activation of estrogen responsive promoters containing an activator protein-1 site, with ERα activating and ERβ attenuating transcription.15,16

As might be predicted from their known functions as steroid hormone receptors and transcription factors, ERs mediate estrogen-induced COX-1 and eNOS upregulation and estrogen-induced adhesion molecule downregulation in cultured endothelial cells.6-8,9 In addition, there is evidence that ERs are critically involved in the nongenomic actions of estrogen in endothelial cells; this evidence includes a study indicating that ERα plays a key role in eNOS activation by the hormone.17 Further studies have indicated that the latter processes involve a subpopulation of ERα associated with plasma membrane caveolae.18-20 Recent work in transgenic mice in which either ERα or ERβ expression has been disrupted has also revealed that ERα, and not ERβ, mediates the long-term effects of estrogen on basal endothelial NO production in intact vasculature.21,22 Thus, a significant amount of knowledge is accumulating regarding the diverse
roles of ERs (in particular, ERα) in the vascular endothelium. In contrast, the mechanisms that regulate ERα expression in the endothelium are poorly understood.

To better understand the processes by which ERα abundance is modified in the vascular endothelium, the present investigation was designed to determine the direct effect of estrogen on the expression of the receptor in cultured endothelial cells. We raised the hypothesis that estrogen upregulates ERα expression in endothelial cells. In addition to testing this hypothesis, experiments were performed to address the following questions: (1) What is the temporal nature of estrogen action on endothelial ERα expression? (2) Does estrogen have comparable effects on endothelial ERα and ERβ expression? (3) Is the effect of estrogen on ERα expression transcriptionally based? (4) Is the effect of estrogen on endothelial ERα expression mediated through ERα and/or ERβ?

Materials and Methods

Cell Culture and Treatment

Primary endothelial cells were obtained from the intrapulmonary arteries of mixed-breed fetal sheep at 125 to 135 days of gestation, with term being 144±4 days, using methods that we have previously described.23 These cells were chosen because they continue to faithfully express ERα and ERβ in cell culture.8,13 Cells from four different primary cultures were studied at passages 4 to 6. Before study, nearly confluent cells were placed in phenol red-free serum-free medium to remove the effects of the estrogen-like activity of phenol red and estrogen derived from the serum. The procedures followed for the care and use of the sheep were approved by the Institutional Animal Care and Research Advisory Committee of the University of Texas Southwestern Medical Center.

To determine the dose response to estrogen, cells were treated with vehicle or 17β-estradiol (E2, 10−14 to 10−9 mol/L) every 18 hours and harvested at 48 hours. To further evaluate the time course of the effect of E2, cells were treated once with vehicle or with 10−8 mol/L E2 and harvested at 6, 12, and 24 hours. In additional experiments, cells were treated with E2 and harvested at 2, 4, and 6 hours. E2 stock solutions were prepared in ethanol (1 mg/mL), and appropriate dilutions of ethanol served as vehicle controls. Studies with vehicle alone versus medium alone showed a transient decline in ERα expression that resolved by 12 hours. The effects of nonselective ER antagonist were assessed using ICI 182,780 (10−5 mol/L), and the role of gene transcription was determined using actinomycin D (25 μg/mL).17 ICI 182,780 did not alter ERα protein abundance in the absence of estrogen. Nor did actinomycin D modify ERα expression in the absence of ligand, yielding abundance that was 120±25% (mean±SEM, n=5) of control values.

Immunoblot Analyses

Immunoblot analyses were performed using methods modified from those previously reported.24 Cells were harvested in ice-cold PBS containing 138 mmol/L NaCl, 2.67 mmol/L KCl, 1.47 mmol/L KH2PO4, and 8.10 mmol/L Na2HPO4; they were pelleted and resuspended in 50 mmol/L Tris buffer (pH 7.4) containing 16 mmol/L CHAPS, 100 mmol/L NaCl, 0.5 mmol/L EDTA, 0.02 mmol/L EGTA, 0.4 mmol/L β-mercaptoethanol, 1.6 mmol/L dithiothreitol, and 2 μg/mL each of soybean trypsin inhibitor, lima bean trypsin inhibitor, antipain, and leupeptin; and then they were ultrasonically disrupted (Branson Ultrasound). The protein content of the preparations was determined by Bradford assay,25 and SDS-PAGE was performed on equal protein samples (40 μg each). Coomassie blue staining was used to confirm equal protein loading. The proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Millipore Corp) over 2 hours on ice, and the membranes were blocked for 1 hour at room temperature in buffer containing 137 mmol/L NaCl and 20 mmol/L Tris (pH 7.5) with 0.5% Tween 20 and 5% dried milk and incubated overnight at 4°C with monoclonal antibody to ERα (AER320, 2 μg/mL, NeoMarkers, Inc) or polyclonal antibody to ERβ (1 μg/mL, Affinity Bioreagents Inc). ERα- and ERβ-positive controls consisted of lysates of COS-7 cells transfected with human ERα or murine ERβ cDNA. The membranes were washed three times and incubated with secondary antibody for 1 hour. The membranes were again washed, and signal for ERα or ERβ protein was visualized by chemiluminescence (ECL Western Blotting Analysis System, Amersham). Amido black staining was used to confirm equivalence of the protein loads on the membranes.

ERα Promoter Activity

The human ERα gene promoter P-1 (~900 to 212 relative to the major transcription start site) was kindly provided by Dr Myres Brown (Dana Faber Institute, Boston, Mass).26 P-1 was cloned into the pGL2 luciferase reporter gene (ERα~900/+212-LUC) using standard techniques. Cell transfection was performed using methods modified from those previously reported.8 Cells grown to 60% to 70% confluence in 6-well plates were washed once with OptiMEM medium (Life Technologies, Inc) and preincubated in OptiMEM medium for 20 minutes at 37°C. E2~900/+212-LUC and a plasmid containing SV~40–driven β-galactosidase (pSV~40–β-gal, Promega Corp) to normalize for transfection efficiency were mixed (1 μg per well of each) with LipofectAMINE (10 μL per well, Life Technologies, Inc) and incubated in a total volume of 200 μL for 30 minutes at room temperature. The lipoid-coated DNA and 800 μL of Opti-MEM were added to each well of cells. After 3 hours, 2 mL medium was added. In selected wells, the cells were alternatively transfected with pGL2-Control Vector (Promega Corp) containing an SV~40 promoter and enhancer or with pGL2-Basic Vector lacking the SV~40 promoter and enhancer (Promega Corp), serving as positive and negative controls, respectively. Twenty-four hours after transfection, the cells were washed in phenol red-free serum-free medium and placed in phenol red-free medium containing 10% charcoal-stripped serum. The charcoal stripping removes estrogen metabolites and other steroid hormones. The cells were treated for 2 to 48 hours with control medium or medium containing 10−8 mol/L E2, with repeat treatments every 18 hours. The cells were lysed, and the extracts were centrifuged at 12 000g to remove unbroken cells and debris. Luciferase activity was measured using a luminometer (Moonlight 2010, Analytical Luminescence Laboratory).27 and β-galactosidase activity was determined spectrophotometrically (at 420 nm) by the generation of o-nitrophenol from the substrate o-nitrophenyl-β-D-galactopyranoside.28 The results were normalized as relative luciferase light units/β-galactosidase activity.

The role of ER in the regulation of promoter activity was determined by treatment with the nonselective ER antagonist ICI 182,780 (10−5 mol/L) or RR-tetrahydrochrysene (THC, 10−5 mol/L), which is a selective ERβ antagonist and an ERs agonist in certain paradigms.29 THC was the kind gift of John and Benita Katzenellenbogen (University of Illinois at Urbana-Champaign College of Medicine, Chicago, Ill). To determine the role of ER subtypes in an additional independent manner, studies were performed in endothelial cells cotransfected with either sham plasmid or human ERα or murine ERβ cDNA.

Statistical Analysis

Comparisons between two groups were made by nonpaired Student t tests. Findings in ≥3 groups were assessed by ANOVA and Newman-Keuls post hoc testing. Results are expressed as mean±SEM, and all stated differences achieved statistical significance at P<0.05.

Results

Effect of E2 on ERα Expression

To first determine whether estrogen modifies ERα expression in endothelial cells, the effects of varying concentrations of E2 were evaluated over 48 hours, with repeat treatments with E2.
Figure 1. Physiological concentrations of E2 upregulate ERα expression in endothelial cells. Cells were treated with vehicle (control [CON]) or 10^{-14} to 10^{-8} M E2 for 6 hours. ERα protein was detected at 67 kDa, as shown in panel A. The immunoblot shown is representative of 4 independent experiments. Cumulative results for quantitative densitometry for the 4 experiments are shown in panel B. Mean±SEM values are depicted for protein abundance expressed as percentage in CON-treated cells. *P<0.05 vs control.

The time course for the change in ERα protein expression after a single treatment with E2 is presented in Figure 2. The time-course experiments for 6 to 24 hours (Figures 2C and 2D) were performed first, and the observations obtained prompted additional experiments focused on time points up to 6 hours (Figures 2A and 2B). In the representative immunoblot shown for the early time course (Figure 2A), compared with vehicle, E2 caused a decline in ERα protein abundance at 2 hours; there was no difference in control or treated samples at 4 hours; and the increase was no longer evident at 24 hours. Cumulative results from four studies revealed 2.7- and 2.5-fold increases in receptor abundance at 6 and 12 hours of E2, respectively, and no change from control cells at 24 hours (Figure 2D).

Role of ER Activation in ERα Modulation by E2
The importance of ER activation in changes in ERα expression induced by E2 in endothelial cells was evaluated using ICI 182,780, which is a nonselective antagonist of ERα and ERβ. The representative immunoblot shown (Figure 3A), 48 hours of treatment with E2 caused an increase in ERα protein abundance, and the upregulation was prevented by ICI 182,780. Cumulative findings from four studies revealed a 3.6-fold increase in ERα protein with E2 and complete reversal of this effect by ER antagonism (Figure 3B).

Effect of E2 on ERβ Expression
To determine whether the findings for E2 modulation of ERα expression in endothelial cells are unique to that ER subtype, parallel studies of ERβ expression were performed (Figure 4). In contrast to the upregulation in ERα noted after 48 hours of exposure to E2, there was a decline in ERβ protein abundance (Figure 4A). The effect of E2 on ERβ expression was reversed by nonselective ER antagonism with ICI 182,780. Repeated studies demonstrated a 56% decline in ERβ protein expression with long-term E2 treatment (Figure 4B). In contrast, E2 exposure for 2, 4, 6, or 12 hours did not alter ERβ expression, with abundance observed to be 104±9%, 111±14%, 98±9%, and 99±22% of control values (mean±SEM, n=4) for the same time points, respectively.

E2 and ERα Gene Transcription in Endothelium
To determine the role of gene transcription in ERα modulation by E2, studies were performed with actinomycin D. In the
representative experiment shown, E₂ treatment for 48 hours yielded an increase in ERα protein, and this was completely prevented by actinomycin D (Figure 5A). Summary results for three separate studies confirmed these observations (Figure 5B), indicating a 615% increase in ERα protein. Parallel studies of ERα or ERβ expression were performed at 48 hours after retreatment of the cells to determine whether the observations for endothelial ERα are unique to that receptor subtype. In contrast to the findings for ERα, initial expression is mediated. In the present study, we determined the direct impact of the hormone on the expression of the receptor in cultured endothelial cells. We demonstrate that long-term exposure to physiological levels of E₂ causes ERα protein upregulation, indicating that the ligand has potent effects on the expression of its own receptor in endothelium.

We observed that the initial (2-hour) exposure to E₂ causes a decline in endothelial ERα protein abundance. This finding parallels the fall in ERα levels initially described in the uterus after estrogen administration to rats and in cultured uterine cells and breast cancer cell lines after estrogen exposure. More recent work in transfected cells has demonstrated that ERα is degraded by the ubiquitin-proteasome pathway in a hormone-dependent manner and that such degradation is actually required for Erα to serve as a transcription factor.

The role of ERs in endothelial cells is quite rapid, and it leads to receptor levels that are considerably greater than baseline. The studies performed at 48 hours after treatment of the cells indicate that persistently elevated levels of the ligand will maintain the enhanced endothelial ERα expression. Collectively, the time-course experiments indicate that the regulation of ERα expression in endothelial cells is a dynamic process that is biphasic if exposure to fresh ligand is persistent.

The role of ERs in endothelial ERα regulation was then determined using the ER-subtype nonselective antagonist ICI 182,780. Whereas long-term exposure to E₂ caused Erα upregulation, concomitant treatment with ICI 182,780 completely prevented the change in receptor abundance. This is the first direct evidence in any cell type of a role for the receptor in the regulation of its own expression at endogenous constitutive levels. As such, there is potential amplification of ERα action in endothelial cells with prolonged ligand activation.

Parallel studies of ERβ expression were performed to determine whether the observations for endothelial ERα are unique to that receptor subtype. In contrast to the findings for ERα, initial

**Discussion**

Estrogen has important effects on both nongenomic and genomic processes in the vascular endothelium, and many of these effects occur through ERα activation. However, little is known about the processes whereby endothelial ERα

**Figure 3.** ERs modulate the E₂-induced increase in ERα expression. Cells were treated with vehicle (CON), 10⁻⁸ mol/L E₂ alone, or 10⁻⁸ mol/L E₂ plus 10⁻⁵ mol/L ICI 182,780 (ICI) for 48 hours. ERα protein was detected at 67 kDa, as shown in panel A. The immunoblot shown is representative of 4 independent experiments. Cumulative results for quantitative densitometry for the 4 experiments are shown in panel B. Mean±SEM values are depicted for protein abundance expressed as percentage in CON-treated cells. *P<0.05 vs CON; †P<0.05 vs E₂ alone.

**Figure 4.** E₂ downregulates ERβ expression in endothelial cells. Cells were treated with vehicle (CON), 10⁻⁸ mol/L E₂ alone, or 10⁻⁸ mol/L E₂ plus 10⁻⁵ mol/L ICI 182,780 (ICI) for 48 hours. ERβ protein was detected at 54 kDa, as shown in panel A. The immunoblot shown is representative of 4 independent experiments. Cumulative results for quantitative densitometry for the 4 experiments are shown in panel B. Mean±SEM values are depicted for protein abundance expressed as percentage in CON-treated cells. *P<0.05 vs CON; †P<0.05 vs E₂ alone.

**Figure 5.** A. Representative results for quantitative densitometry for the 4 experiments are shown in panel B. Mean±SEM values are depicted for protein abundance expressed as percentage in CON-treated cells. *P<0.05 vs CON; †P<0.05 vs E₂ alone.
E2 treatment did not modify ERβ abundance, whereas there was marked downregulation of ERβ on long-term exposure to the hormone. The latter process was also ER dependent. This is the first evidence of contrasting ER isoform regulation by the hormone. On the basis of these observations, it is apparent that the net effect of E2 on endothelial cell signaling and/or gene expression represents the combination of potential overlapping and contrasting ERα and ERβ actions in that cell type and also the opposing dynamics of ER-subtype expression. These considerations are important to the interpretation of findings in model systems using gain-of-function or loss-of-function strategies to delineate the roles of specific ER subtypes in the vascular wall.

The basis for E2-induced changes in ERα protein levels in the endothelium was then investigated using actinomycin D to inhibit gene transcription. Whereas the agent did not alter ERα abundance in the absence of hormone as ligand, actinomycin D completely prevented E2-related increases in the abundance of the receptor. Consistent with this observation, the activity of the human ERα promoter transfected in endothelial cells was increased by E2 in an ER-dependent manner. Prior studies of the promoter in MCF7 or HeLa cells have shown that estrogen inducibility is mediated by three half-estrogen response elements,26 and similar processes are likely to be active in endothelial cells. Importantly, it was possible to demonstrate robust activation of the ERα promoter within 4 hours of E2 treatment, thus preceding the upregulation in protein expression observed at 6 hours and beyond. The activation of ERα gene transcription and the resultant upregulation in the protein in endothelial cells is extremely rapid compared with those in other endothelial cell genes that are regulated by the hormone, such as COX-1 and eNOS, whose expressions are not altered before 48 hours of hormone exposure.6,8

Additional experiments were performed to determine the receptor subtype responsible for ERα gene activation in endothelial cells. In cells cotransfected with the ERα promoter-reporter gene construct and cDNAs for either ERα or ERβ, the overexpression of either receptor subtype yielded greater promoter activation by E2, indicating that both ERα and ERβ are capable of mediating ERα gene expression. These findings were confirmed in studies using the ERβ-selective antagonist THC, which attenuated approximately half of E2-induced ER-dependent promoter activation. Thus, the regulation of ERα expression in endothelial cells entails both homologous and heterologous sensitization.

The current observations add new insights to our prior understanding of the modulation of endothelial ER by estrogen. Previous studies that were limited to determinations of ERα mRNA levels in human umbilical vein endothelial cells demonstrated an increase in the mRNA after 48 hours of hormone
exposure, and this is consistent with the present findings. It has been shown with the use of radioligand binding assays that rat liver sinusoidal endothelial cell ER abundance declines after ovariectomy and increases after ethinyl estradiol treatment. However, studies in whole mouse aortas have revealed greater E2 binding sites in males versus females, and no changes were detected after long-term estrogen treatment. In addition, ERα and ERβ expression in rat uterine endothelium evidently does not vary during the estrous cycle, but these observations were exclusively dependent on immunohistochemical approaches. In contrast to these prior studies, the present work directly quantifies both ERα and ERβ protein levels in endothelial cells. Although the current in vitro model system is potentially limited by the use of a single endothelial cell type removed from physiological conditions, such an approach has made it possible to determine the direct effects of E2 on endothelial ER expression and to delineate the basic mechanisms underlying the changes in ERα abundance. Further studies are now warranted using receptor-subtype–specific quantitative measures in intact animal models to determine how estrogen alters endothelial ERα and ERβ expression in multiple vascular beds.

The physiological and pathophysiological implications of the present findings are many. Consistent with the apparent complexities in the temporal nature of ER regulation in the vascular endothelium, prior studies of estrogen-induced vascular responses have yielded a variety of time courses of hormone effect. As suggested previously by Parker et al., there are at least three patterns of response. There are rapid vasodilator responses within 5 minutes, such as those observed in isolated coronary artery rings from human adult males. In contrast, vasodilation in the uterine arteries of nonpregnant ewes is delayed for 40 minutes and peaks at 100 minutes before falling back to baseline over several hours. Other models display delayed vasodilator responses that occur after days of hormone exposure, such as the effect of E2 on the pulmonary circulation of the ovine fetus. Whereas the temporal characteristics of the vascular responses to estrogen may be due to multiple mechanisms, it is postulated that initial endothelial ER degradation and ultimate ERα upregulation or ERβ downregulation may be involved. In addition, conditions of estrogen deficiency, such as surgical or natural menopause, may be characterized by perturbed endothelial ER expression, thereby potentially contributing to the increase in vascular disease associated with those states. Future directed studies of the regulation of ERα and ERβ expression in the endothelium will increase our basic understanding of the mechanisms by which estrogen has an impact on vascular health.

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