Cell-Surface Estrogen Receptors Mediate Calcium-Dependent Nitric Oxide Release in Human Endothelia

George B. Stefano, PhD; Vincent Prevot, PhD; Jean-Claude Beauvillain, PhD, MD; Patrick Cadet, PhD; Caterina Fimiani, MD; Ingeborg Welters, MD; Gregory L. Fricchione, MD; Christophe Breton, PhD; Philippe Lassalle, PhD; Michel Salzet, PhD; Thomas V. Bilfinger, MD

Background—Although estrogen replacement therapy has been associated with reduction of cardiovascular events in postmenopausal women, the mechanism for this benefit remains unclear. Because nitric oxide (NO) is considered an important endothelium-derived relaxing factor and may function to protect blood vessels against atherosclerotic development, we investigated the acute effects of physiological levels of estrogen on NO release from human internal thoracic artery endothelia and human arterial endothelia in culture.

Methods and Results—We tested the hypothesis that estrogen acutely stimulates constitutive NO synthase activity in human endothelial cells by acting on a cell-surface receptor. NO release was measured in real time with an amperometric probe. 17β-Estradiol exposure to internal thoracic artery endothelia and human arterial endothelia in culture stimulated NO release within seconds in a concentration-dependent manner. 17β-Estradiol conjugated to bovine serum albumin also stimulated NO release, suggesting action through a cell-surface receptor. Tamoxifen, an estrogen receptor inhibitor, antagonized this action. We further showed with the use of dual emission microfluorometry that 17β-estradiol–stimulated release of endothelial NO was dependent on the initial stimulation of intracellular calcium transients.

Conclusions—Physiological doses of estrogen immediately stimulate NO release from human endothelial cells through activation of a cell-surface estrogen receptor that is coupled to increases in intracellular calcium. (Circulation. 2000;101:1594-1597.)

Key Words: nitric oxide ■ hormones ■ calcium ■ endothelium
supplemented with 10% fetal calf serum and endothelial growth factor at 37°C in 5% CO₂.¹⁵

Direct Measurement of NO Release

NO release from HAEC (10⁶ cells/chamber) and ITA fragments (3-mm rings) was measured directly with the use of an NO-specific amperometric probe (World Precision Instruments).⁷ Each experiment was repeated 4 times and was simultaneously performed with a control from the same tissue source (vehicle alone). Data were evaluated by a Student’s 𝑡 test after acquisition by a computer-interfaced DUO-18 software (World Precision Instruments).

To evaluate NO release, cells were exposed to a concentration gradient of the various ligands. If an antagonist or a NOS inhibitor, Nω-nitro-L-arginine methyl ester (L-NAME), was used, it was administered 5 minutes before that of the various estrogen ligands.

Ligands

Tissues were stimulated with various concentrations of 17β-estradiol or 17β-estradiol conjugated to bovine serum albumin (E₂-BSA). To determine that there was no dissociation between 17β-estradiol and BSA, an RIA kit optimized for the direct quantitative determination of 17β-estradiol was used (ICN kit). 17β-Estradiol measured in the cytosolic fraction of HAEC treated with 10⁻⁹ and 10⁻⁸ mol/L E₂-BSA revealed no estradiol in the cytosol (assay sensitivity was 0.2 pg/tube). All drugs were purchased from Sigma Chemical Co (St Louis) except ICI 182,780, which was kindly provided by Zeneca Pharmaceuticals.

Intracellular Calcium Imaging

Intracellular calcium levels were measured in HAEC in culture by dual emission microfluorometry with the fluorescent dye fura-2/AM.¹⁵ Images were acquired every 0.4 second with an image processing system Compix C-640 SIMCA (Compix Inc) and an inverted Nikon microscope. The respective receptor antagonists were administered 2 minutes before the respective agonist. Control [Ca²⁺]ᵢ “sparkling” is in the 0 to 3 nmol/L range.

A 2-way ANOVA was used for statistical analysis on the peak [Ca]ᵢ time, 7 seconds after agonist exposure to the cells. Each experiment was simultaneously performed with up to 8 cells. The mean value was combined with the mean value taken from 4 other replicates.

Reverse Transcription–Polymerase Chain Reaction Analysis

Total RNA from human umbilical vein endothelial cells (HUVEC) was extracted with Trizol (Gibco/BRL) and reverse-transcribed into cDNA with the use of random hexamers and Moloney Murine Leukemia Virus RT (Gibco/BRL).¹⁶ For ERα amplification, the primer pair (25-mers) was designed to amplify a 281-bp fragment

Figure 1. Real-time representation of 17β-estradiol–stimulated (E, 10⁻⁹ mol/L) NO and [Ca]ᵢ from HAEC. A, 17β-estradiol–stimulated NO release and (bottom) its (E) antagonism by prior tamoxifen (T, 10⁻⁹ mol/L) exposure. B, Representative real-time calcium transients from HAEC. Top, Addition of 17β-estradiol (E, 10⁻⁹ mol/L), at base of determination, results in 47 nmol/L [Ca]ᵢ. Middle, Addition of tamoxifen (T, 10⁻⁹ mol/L, straight line raised for better visualization) blocks the 17β-estradiol–stimulated [Ca]ᵢ (by E). Bottom, ICI 182,780 (ICI, 10⁻⁹ mol/L) addition to the medium 2 minutes before that of 17β-estradiol–stimulated [Ca]ᵢ (at E).

Figure 2. Dose-dependent release of NO after in vitro stimulation of (A) ITA fragments or (B) HAEC (10⁶ cells/mL) by 17β-estradiol and E₂-BSA. Graphed values represent peak values obtained 2 minutes after drug exposure. Cells were exposed to agents for entire observation period (15 minutes; see Figure 1). Each experiment was repeated 4 times; resulting mean value (±SEM) was graphed.

Figure 3. 17β-Estradiol stimulates NO release through membrane receptor. 17β-Estradiol (17 B-E) and E₂-BSA stimulate NO release from ITA fragments (A) and HAEC (B) in tamoxifen-sensitive process. Each experiment was repeated 4 times; resulting mean value (±SEM) was graphed.

Figure 4. E₂-BSA stimulation of NO release from (A) ITA or (B) HAEC is not antagonized by low concentrations of ICI 182,780 (ICI; 10⁻⁹ mol/L). Each experiment was repeated 4 times; resulting mean value (± SEM) was graphed.
Results

In real time, 17β-estradiol, in a dose-dependent manner, stimulated the release of NO (16.1 ± 2.7 × 10⁻⁶ mol/L peak value), well above the low level (0 to 1 nm) of constitutive release (Figure 1A and Figure 2). Tamoxifen (10⁻⁹ mol/L) significantly diminished (P < 0.005) 17β-estradiol–stimulated NO release (Figure 1 and Figure 3). 17α-Estradiol (10⁻⁹ mol/L) did not stimulate NO release from either tissue (data not shown).

ICI 182,780, another estrogen receptor antagonist, did not effect NO release from either type of endothelial cell in the 10⁻¹² to 10⁻⁷ mol/L range (Figure 1 and Figure 4) but did at 10⁻⁶ mol/L, reducing NO release by 78%. Neither tamoxifen (10⁻¹⁰ to 10⁻⁷ mol/L) nor ICI 183,780 (10⁻⁷ to 10⁻⁵ mol/L) had agonistic effects on NO release (Figure 1 and Figure 4).

17β-Estradiol Acts at a Surface Receptor

E₂-BSA, which does not penetrate the cellular membrane, also stimulates NO release in a dose-dependent, tamoxifen-sensitive manner (Figure 2 Figure 4, and Figure 5). Additionally, in ITA gently scraped to remove the endothelial lining, 17β-estradiol nor E₂-BSA stimulated NO release from the remaining tissues (data not shown), demonstrating an estrogen cell-surface receptor. In addition, L-NAME (100 μmol/L) blocked the NO-stimulating activities of both 17β-estradiol and E₂-BSA in both cell types (Table).

Discussion

The present study demonstrates that at physiological concentrations, 17β-estradiol rapidly stimulates NO release from human ITA and HAEC. This process is mediated by a specific estradiol receptor, as noted by its antagonism by tamoxifen. The E₂-BSA stimulation of NO release indicates that this receptor is located on the cell surface. Furthermore, estrogen agonist-stimulated NO release is inhibited by L-NAME, indicating that NO release is mediated by coupling the cell-surface estrogen receptor to eNOS. Additionally, estrogen-stimulated release of endothelial NO is dependent on the initial stimulation of [Ca], supporting the eNOS activation by estrogen. Taken together, our data and those of others demonstrate the existence of a cell-surface receptor for estrogen and its coupling to NO by [Ca]. This finding might

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<th>L-NAME Inhibits Estrogen-Stimulated NO Release</th>
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<tr>
<td>Cells</td>
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<td>E-culture 17β-estradiol</td>
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Each experiment was replicated 4 times.

P < 0.01, NO release in presence of L-NAME vs that in appropriate tissues without it.
explain some beneficial actions of estrogens, for instance Short-term effects observed in premenopausal women.6,20 This rapid action of estrogen at a cell-surface receptor is in contrast with its known long-term (after 8 hours), ICI-182,780–sensitive action through intracellular receptors, effecting NO release from endothelia.9,21

Previous studies have shown that estradiol increases intracellular calcium levels and activates NO release in a tamoxifen-sensitive and ICI-182,780-sensitive manner, purportedly through both intracellular12 and nonintracellular receptors.13 We present the strongest evidence for the existence of a cell-surface–mediated pathway: Although the estrogen receptor ligand E2-BSA stimulates calcium-dependent NO release, it is too large to pass through the cell membrane.

Our results of a cell-surface estrogen receptor are further supported by studies that show that cells expressing ERα and ERβ target the protein to both membrane and nuclear fractions.14 Although our RT-PCR results suggest the presence of only ERβ transcripts in human endothelium, we do not know if these are the receptors that mediate NO release in response to 17β-estradiol; recent studies have identified several variant ER transcripts.22 Furthermore, others have demonstrated ERα immunoreactivity in human and monkey coronary artery.23

Finally, our results are supported by other functional studies that demonstrate a rapid-acting vasodilatory role for estrogen-mediated NO release11 and the potential to diminish immunocyte adherence.7 The significance of these processes may correlate with the beneficial activities reported for estrogen in vascular tissues and those pathologies associated with immunocyte activation.7

Acknowledgments
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References
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