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

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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. 17b-Estradiol exposure to internal thoracic artery endothelia and human arterial endothelia in culture stimulated NO release within seconds in a concentration-dependent manner. 17b-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 17b-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

The incidence of cardiovascular events in women increases after menopause, suggesting that estrogen deficiency may play a role in cardiovascular disease. Favorable alterations in serum lipids and decreased vascular reactivity are both thought to contribute to the cardioprotective effect of estrogen. The mechanisms by which estrogen influences coronary arteries and protects blood vessels against atherosclerotic development are unclear, but recent evidence suggests that nitric oxide (NO) production may play an important role.

Estradiol has been shown to increase endothelial constitutive NO synthase (ecNOS) expression through intracellular receptors. However, the rapid effects of estradiol observed on vascular reactivity and on NO release from endothelial cells after short-term estradiol administration are incompatible with transcriptionally mediated pathways. Two recent reports showed that 17β-estradiol mediates nongenomic activation of ecNOS in cultured endothelial cells and that α- and β-estrogen receptors (ER) localize to both nuclear and membrane fractions. The purpose of our study was to investigate the existence of cell surface estrogen receptors that mediate acute activation of ecNOS by estrogen in human endothelia.

Methods

Materials

Internal thoracic artery (ITA) segments were obtained from patients (4 postmenopausal women, mean age 74.5±10.3 years, and 8 men mean age 57.6±13.3 years) undergoing elective coronary artery bypass grafting (CABG) for atherosclerotic coronary artery disease. This material is regarded as waste, and the institutional review board approved the project. Patients with chronic illnesses, for example, diabetes, were excluded. ITA specimens were prepared as previously described.

Human arterial endothelial cells (HAEC) purchased from Cell Systems (Eugene, Ore) were grown in chamber slides (Nunc Int) with the use of CS-C medium (phenol red free; Cell Systems).
supplemented with 10% fetal calf serum and endothelial growth factor at 37°C in 5% CO₂.

**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). 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. 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). 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.
L-NAME Inhibits Estrogen-Stimulated NO Release

<table>
<thead>
<tr>
<th>Cells</th>
<th>Estrogen Agonist, (10^{-9}) mol/L</th>
<th>L-NAME, (\mu)mol/L</th>
<th>NO Level, nmol/L ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-culture</td>
<td>17β-estradiol</td>
<td>...</td>
<td>13.8 ± 1.2</td>
</tr>
<tr>
<td>E-culture</td>
<td>17β-estradiol</td>
<td>100</td>
<td>2.3 ± 0.8</td>
</tr>
<tr>
<td>ITA</td>
<td>17β-estradiol</td>
<td>...</td>
<td>14.6 ± 2.1</td>
</tr>
<tr>
<td>ITA</td>
<td>17β-estradiol</td>
<td>100</td>
<td>2.7 ± 0.9</td>
</tr>
</tbody>
</table>

Each experiment was replicated 4 times. 

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 ecNOS. Additionally, estrogen-stimulated release of endothelial NO is dependent on the initial stimulation of [Ca], supporting the cNOS 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
explain some beneficial actions of estrogens, for instance Short-term effects observed in premenopausal women.\textsuperscript{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.\textsuperscript{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 intracellular\textsuperscript{12} and nonintracellular receptors.\textsuperscript{13} We present the strongest evidence for the existence of a cell-surface–mediated pathway: Although the estrogen receptor ligand E\textsubscript{2}-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 ER\textalpha{} and ER\beta{} target the protein to both membrane and nuclear fractions.\textsuperscript{14} Although our RT-PCR results suggest the presence of only ER\beta{} 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.\textsuperscript{22} Furthermore, others have demonstrated ERs immunoreactivity in human and monkey coronary artery.\textsuperscript{23}  
Finally, our results are supported by other functional studies that demonstrate a rapid-acting vasodilatory role for estrogen-mediated NO release\textsuperscript{11} and the potential to diminish immunocyte adherence.\textsuperscript{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.\textsuperscript{7}  

Acknowledgments

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References

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