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

The incidence of cardiovascular events in women increases after menopause, suggesting that estrogen deficiency may play a role in cardiovascular disease.1 Favorable alterations in serum lipids and decreased vascular reactivity are both thought to contribute to the cardioprotective effect of estrogen.2,3 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.4–7

Estradiol has been shown to increase endothelial constitutive NO synthase (eNOS) expression through intracellular receptors.9 However, the rapid effects of estradiol observed on vascular reactivity10,11 and on NO release from endothelial cells12 after short-term estradiol administration are incompatible with transcriptionally mediated pathways. Two recent reports showed that 17β-estradiol mediates nongenomic activation of eNOS in cultured endothelial cells13 and that α- and β-estrogen receptors (ER) localize to both nuclear and membrane fractions.14 The purpose of our study was to investigate the existence of cell surface estrogen receptors that mediate acute activation of eNOS 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.7

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, N⁵-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 10⁻²⁹ and 10⁻²⁸ mol 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 “sparking” 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.
Results

In real time, 17β-estradiol, in a dose-dependent manner, stimulated the release of NO (16.1 ± 2.7 10⁻⁹ 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 (10⁻¹² to 10⁻⁷ 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, neither 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).

Direct Evaluation of Intracellular Calcium Release

In real time, 17β-estradiol (10⁻⁹ mol/L) stimulated a rapid [Ca]i within 6 seconds of its exposure (EC₅₀=5 × 10⁻¹⁰ mol/L) (Figure 1B). This event could be blocked by prior tamoxifen (10⁻⁹ mol/L) (IC₅₀=8 × 10⁻¹⁰ mol/L) treatment but not by ICI 182,780 at this concentration (Figure 1B). After depletion of intracellular calcium stores, 15 17β-estradiol (10⁻⁹ mol/L) increased [Ca]i to 3.8 ± 0.6 nmol/L, a level substantially lower than those under nondepleting conditions (Figure 1). Furthermore, NO release was barely above background (control = 0 to 3.0 nmol/L) in the calcium-depleted HAEC after 17β-estradiol (NO 1.8 ± 0.6 nmol/L compared with a peak value of 16.0 ± 2.7) exposure.

Endothelial Estrogen Receptor Expression

Reverse transcription (RT)-PCR analysis of RNA from HUVEC reveals expression of ERβ (Figure 6, lane 3), the same receptor is expressed in breast cell lines (lane 2). ERα products were not detected. DNA sequencing of the PCR products obtained for human breast cell lines and HUVEC revealed a nucleotide sequence 100% homologous human ERβ.

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 cNOS. 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 only ER\textsubscript{a} supported by studies that show that cells expressing ER\textsubscript{a} membrane.

several variant ER transcripts.\textsuperscript{22} Furthermore, others have reported through both intracellular\textsuperscript{12} and nonintracellular receptors.\textsuperscript{13} We present the strongest evidence for the existence of only ER\textsubscript{a} supported by studies that show that cells expressing ER\textsubscript{a} membrane.

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

This study was supported in part by the following grants: NIMH-17138, -47392, NIDA 09010, NIH Fogarty INT 00045 (Dr Stefano), the University of Lille II, and the FEDER.

References

Cell-Surface Estrogen Receptors Mediate Calcium-Dependent Nitric Oxide Release in Human Endothelia
George B. Stefano, Vincent Prevot, Jean-Claude Beauvillain, Patrick Cadet, Caterina Fimiani, Ingeborg Welters, Gregory L. Fricchione, Christophe Breton, Philippe Lassalle, Michel Salzet and Thomas V. Bilfinger

Circulation. 2000;101:1594-1597
doi: 10.1161/01.CIR.101.13.1594

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/101/13/1594

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/