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Nongenomic Stimulation of Nitric Oxide Release by Estrogen Is Mediated by Estrogen Receptor α Localized in Caveolae

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Acute administration of 17β-estradiol (E_2) exerts antiatherosclerotic effects in healthy postmenopausal women. The vasoprotective action of E_2 may be partly accounted for by a rapid increase in nitric oxide (NO) levels in endothelial cells (ECs). However, the signaling mechanisms producing this rise are unknown. In an attempt to address the short-term effect of E_2 on endothelial NO production, confluent bovine aortic endothelial cells (BAECs) were incubated in the absence or presence of E_2, and NO production was measured. Significant increases in NO levels were detected after only 5 min of E_2 exposure without a change in the protein levels of endothelial NO synthase (eNOS). This short-term effect of estrogen was significantly blunted by various ligands which decrease intracellular Ca^{2+} concentration. Furthermore, plasma membrane-impermeable BSA-conjugated E_2 (E_2BSA) stimulated endothelial NO release, indicating that in the current system the site of action of E_2 is on the plasma membrane rather than the classical nuclear receptor. The partial antagonist tamoxifen did not block E_2-induced NO production; however, a pure estrogen receptor α (ERα) antagonist ICI 182,780 completely inhibited E_2-stimulated NO release. The binding of E_2 to the membrane was confirmed using FITC-labeled E_2BSA (E_2BSA-FITC). Western blot analysis showed that plasmalemmal caveolae possess ERα in addition to well-known caveolae-associated proteins eNOS and caveolin. This study demonstrates that the nongenomic and short-term effect of E_2 on endothelial NO release is Ca^{2+}-dependent and occurs via ERα localized in plasmalemmal caveolae.

Key Words: **estrogen; nitric oxide; nitric oxide synthase; caveolae; nongenomic action.**

Estrogen is one of the best known protectors against the development of coronary heart disease due to its vasodilatory action. Although acute administration of 17β-estradiol (E_2) in healthy postmenopausal women has been widely used, the precise mechanism by which E_2 exerts its beneficial effect on cardiovascular disease is currently under investigation. The vasodilatory effect of E_2 is mostly achieved by the action of endothelium-derived nitric oxide (NO) produced by endothelial nitric oxide synthase (eNOS). The classical action of estrogen via a nuclear receptor, which modulates gene expression, is well established (1–3). However recent evidence suggests that a nongenomic action of estrogen may have an important role in its acute effect on vasodilation (4–6). NO is an important bioregulatory molecule and serves multiple functions including vasodilation and many antiatherogenic properties. eNOS catalyzes the five-electron oxidation of L-arginine to NO by using flavin, tetrahydrobiopterin (BH_4) and Ca^{2+}/calmodulin as important cofactors and NADPH and molecular oxygen as cosubstrates. The regulation of eNOS activity and consequently the regulation of NO signaling occur largely at the level of expression of eNOS. For example, exercise (7), mechanical stimuli (8), hypoxia (9), high glucose (10) and long-term estrogen treatment (11, 12) increase eNOS mRNA and protein levels while tumor necrosis factor α

Abbreviations used: E_2, 17β-estradiol; E_2BSA, 17β-estradiol 6-(*O*-carboxymethyl)oxime:BSA; E_2BSA-FITC, 17β-estradiol 6-(*O*-carboxymethyl)oxime:BSA–fluorescein isothiocyanate conjugate; EC, endothelial cell; BAEC, bovine aortic endothelial cell; NO, nitric oxide; NO_x, reduced NO_2^- and NO_3^-; eNOS, endothelial nitric oxide synthase; ER, estrogen receptor; L-NAME, N^G-nitro-L-arginine methyl ester; BAPTA-AM, 1,2-bis(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; tetrakis(acetoxymethyl ester; PBS, phosphate-buffered saline; HBSS, Hanks' balanced salt solution.

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(13) and high-dose heparin treatment (14) decrease eNOS mRNA posttranscriptionally. In addition to the control of eNOS expression, NO release in the cells is also regulated by other posttranslational modifications of eNOS such as phosphorylation (15) and fatty acylation (16–18). These modifications directly relate to the localization of eNOS within the cells, which might be expected to influence the biological role and chemical fate of the NO produced by the enzyme. In this regard, eNOS is unique among the three known NOS isoforms in being localized to the specialized cell surface signal-transducing domains termed plasmalemmal caveolae in endothelial cells (19). In addition, the interaction between eNOS and caveolin, the most abundant protein in caveolae, greatly attenuates eNOS activity (20). This interaction is regulated by the Ca^{2+}/calmodulin cofactor complex (21). Acute treatment of E_2 increases basal NO release in ECs without elevation of eNOS mRNA and protein (11). However, the molecular mechanism of this acute E_2 action remains unclear and its dependence on either cytosolic Ca^{2+} or nuclear estrogen receptors remains controversial. In addition, the effect of E_2 on the interaction between eNOS and caveolin in the cells is not understood at all. Thus, this study was undertaken to investigate the mechanism by which E_2 acutely increases NO release, without stimulation of nuclear transcription, via the interaction between eNOS and a component in the plasma membrane. We now report, for the first time, that plasmalemmal caveolae-associated ERα mediates the acute E_2-stimulated NO production which occurs via a novel Ca^{2+}-dependent signaling pathway.

MATERIALS AND METHODS

Materials. E_2, E_2BSA, E_2BSA-FITC, L-NAME, progesterone and tamoxifen were purchased from Sigma Chemical Co (St. Louis, MO). Nifedipine, A23187 and BAPTA-AM were obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA) and collagenase (type 2) was from Worthington Biochem. Corp. (Freehold, NJ). The ER antagonist ICI 182,780 was a kind gift from ZENECA Pharmaceuticals (Cheshire, UK). Antibodies against caveolin 1 and eNOS were purchased from Transduction Laboratories (Lexington, KY) and anti-ERα antibody was from Santa Cruz Biotech. Inc. (Santa Cruz, CA). Culture media were purchased from Gibco-BRL (Gaithersberg, MD). All other chemicals were of the purest analytical grade.

Cell culture and E_2 treatment. Bovine aorta, obtained from a local slaughterhouse, was washed in phosphate-buffered saline (PBS) and the luminal side of the aorta was subjected to 0.05% collagenase digestion for 10 min. BAECs were obtained by repeated gentle pipeting on the luminal side and were washed twice by centrifugation at $100 \times g$ for 5 min at 4°C in M199 medium containing 15% fetal calf serum (FCS), 100 U/ml penicillin, 100 μ/ml streptomycin and 1.25 μ/ml amphotericine B. BAECs were serially passaged after treatment with 0.05% trypsin. The ECs were evaluated by their maintenance of density-dependent growth after serial passage by their typical cobblestone configuration when viewed by light microscopy and by a positive indirect immunofluorescence test for von Willebrand factor VIII. Cells between passage 6 and 10 were grown in phenol red-free M199 with dextran-charcoal-stripped 15% FCS and once confluent were treated with E_2 (5 nM) and NO was measured in the absence or presence of various chemicals. In some experiments cells were first pretreated for 30 min either with L-type Ca^{2+} channel blocker nifedipine (20 μM), EGTA (2 mM), BAPTA-AM (10 μM), Ca^{2+} ionophore A23187 (10 μM), or with the ER receptor antagonists tamoxifen (1 μM) or ICI 182,780 (10 μM). In a separate experiment a competitive inhibitor of arginine substrate L-NAME (2 mM) was incubated in modified HBSS solution during NO assay (see below) and in a control experiment, progesterone (10 nM) was used instead of E_2. SK Hep1 cells were purchased from ATCC (Manassas, VA) and propagated in DMEM medium.

NO measurement. Concentrations of NO_x, defined as NO_2^-, and NO_3^-, in culture media were determined by NO-specific chemiluminescence as measured using an Antek nitrate/nitrite reduction assembly and NO analyzer (models 745 and 7020 respectively; Antek Instruments, Inc., Houston, TX). Since this machine measures all the nitrite/nitrate compounds present in the assay medium, including the nitrite/nitrate content of the culture media *per se,* we employed a modified protocol as described before (12). In brief, the medium was changed to modified HBSS (1.2 mM $CaCl_2$, 0.6 mM $MgSO_4$, and 100 μM L-arginine) after treatment and equilibrated for 1 h at 37°C. At the end of the incubation period, supernatants were collected for NO_x analysis and reduced with 1 M NaOH in glacial acetic acid and/or 0.1 M $VOSO_4$ in 2 M HCl before measurement. The NO analyzer detected NO using V^{3+} to chemically reduce the NO_x, the stable end products of NO released from the cells. Free NO was then detected in a chemiluminescence spectrometer by its reaction with ozone with $NaNO_2$ used as the reference standard. All measured NO was expressed as mean (fold) ± SD. The detection limit of NO was 1 pmol. With this protocol, the NO measured represents that which is produced during the 60 min incubation in HBSS following from the agonist/antagonist treatments of specified duration. Subsequent NO production is then solely dependent on eNOS activity at the end of the agonist/antagonist treatments, given that the assay medium contains excessive amounts of cofactors and substrates.

Fluorescence microscopy. Cells were grown on polylysine-coated coverslips and fixed with 4% paraformaldehyde. Fixed cells were incubated with E_2BSA-FITC (20 μM) for 40 min at 4°C after blocking with 0.2% BSA. BSA-FITC alone was used as the corresponding control. The cells were then extensively washed in fresh PBS. The localization of E_2BSA-FITC was analyzed using a Zeiss IM 35 microscope (Zeiss, Oberkochen, Germany).

Western blot analysis. Preparation of detergent-free caveolae from ECs was carried out as previously described (22). Caveolar proteins (50 μ) were separated using sodium dodecyl sulfate (SDS)-polyacrylamide gels followed by electrophoretic transfer onto nitrocellulose membranes. The blots were then probed with polyclonal anti-eNOS (1:1,000), anti-ERα (1:2,500) or anti-caveolin 1 (1:2,000) antibodies and finally developed using enhanced chemiluminescence reagents (ECL, Amersham, DE).

Statistical analysis. Results were expressed as means ±SD with n indicating the number of experiments. Statistical significance of the data was determined by Student's unpaired *t* test where a value of $p < 0.05$ was considered statistically significant.

RESULTS

Physiologically relevant concentrations of E_2 increase NO in BAECs after 5 min without upregulation of eNOS. When BAECs were treated with various concentrations (100 pM to 10 μM) of E_2, a maximal increase in NO levels was observed at 1–10 nM (data not shown). Accordingly, all subsequent experiments were performed using 5 nM E_2. In kinetic experiments as shown in Fig. 1, marked augmentation of NO release was demonstrated at the earliest time point (5 min; by

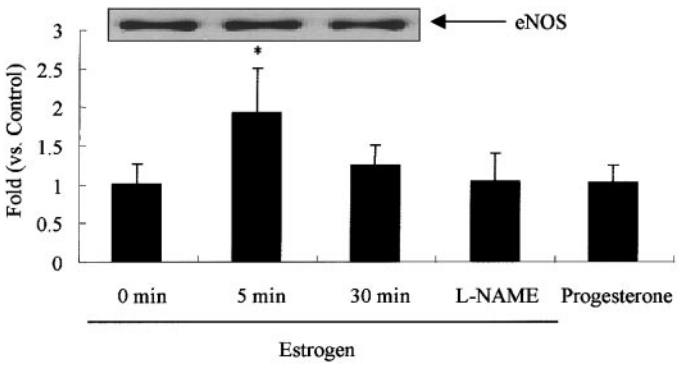

FIG. 1. The short-term effect of E_2, L-NAME, and progesterone on NO release and on eNOS expression in BAECs. Cells were treated in the absence or presence of L-NAME (1 mM) for 30 min prior to E_2 (5 nM) exposure for either 5 min or 30 min and then incubated in modified HBSS for 1 h. In a control experiment, cells were treated with progesterone (10 nM) instead of E_2. Released NO was then measured and cell proteins were processed for Western blot analysis. The basal level of NO was 7.87 ± 1.15 nmol/10^6 cells. Data represent means ± SD from at least three independent experiments. *$p < 0.05$ (vs. control).

1.93 ± 0.58 fold as compared with control). This was shown to be an acute affect as NO levels subsequently fell over the next 30 min period. Western blot analysis revealed that this short-term effect of E_2-mediated NO release did not result from an increase in eNOS protein expression, suggesting that the classical intracellular genomic action is not responsible for the observed effect. The increase in NO by estrogen was blocked when using non-degradable arginine, L-NAME, as a substrate during the assay. Furthermore, progesterone (10 nM), a structurally-related hormone, did not augment NO production at all in the cells.

The short-term effect of E_2 on NO release is Ca^{2+}-dependent. E_2 has been shown to facilitate Ca^{2+} entry and mobilization in certain ECs (23). In addition, intracellular Ca^{2+} is known to regulate eNOS activity, which in turn activates NO release (24). However, it is also reported that E_2 stimulates a rapid NO increase in human umbilical vein ECs, independent of cytosolic Ca^{2+} mobilization (12). From these controversial findings, we have reevaluated if Ca^{2+} is required to increase NO production by acute treatment of E_2. Table 1 shows that the Ca^{2+}-ionophore A23187 (10 μM) effected an increase in NO production comparable to that caused by acute E_2 challenge. Cotreatment of E_2 and A23187 produced the same increase in NO levels as for A23187 alone. In contrast, pretreatment of the cells with the L-type Ca^{2+}-channel blocker nifedipine (20 μM) inhibited E_2-induced NO accumulation. Furthermore, either depletion of extracellular Ca^{2+} by EGTA (2 mM) or intracellular Ca^{2+} by BAPTA-AM (10 μM) completely inhibited the acute response to E_2. Taken together, the short-term effect of E_2 on NO production requires increased intracellular Ca^{2+} levels.

Caveolae-associated ERα mediates the acute increase in NO by E_2. Next we tested whether E_2-induced NO production is mediated by direct binding of E_2 to putative estrogen binding components on the plasma membrane. To this end, we employed the membrane-impermeable estrogen analogue, E_2BSA, which is unable to bind classical intracellular ERs (25) as it cannot enter the cell. Table 1 shows that acute E_2BSA treatment increased NO production following a 5 min incubation period, which is consistent with its acting independently of intracellular receptors. The existence of putative binding components in the plasma membrane was confirmed by fluorescence microscopy. Figure 2 shows that when BAECs were treated with E_2BSA-FITC (1 μM), substantial fluorescence was exhibited at the cell surface (Fig. 2a). In control experiments, no fluorescence was observed on E_2BSA-FITC-treated SK Hep1 cells, an ERα deficient cell-line (Fig. 2b) and also no fluorescence was observed when BAECs where treated with BSA-FITC (data not shown). To further characterize the estrogen-binding components of the plasma membrane we studied the effects of estrogen antagonists on E_2 treatment. Pretreatment with tamoxifen (1 μM), a partial ERα antagonist, did not inhibit E_2-stimulated endothelial NO production (Table 1). However, a pure ERα antagonist ICI 182,780 (10 μM) completely attenuated E_2-induced endothelial NO formation (Table 1). The complete inhibition of E_2-stimulated NO production with ICI 182,780 prompted us to identify whether EC plasma membranes contain the ICI 182,780 binding protein ERα. As shown in Fig. 3, we demonstrated by Western blot analysis that ERα, like eNOS and caveolin 1, is highly enriched in caveolae, being barely observed in bulk plasma membranes (data not shown).

TABLE 1

Effect of E_2 on NO Release from BAECs

Experimental condition	NO production
Control	1.00 ± 0.27 (6)
E_2	1.93 ± 0.58* (10)
A23187	2.07 ± 0.15* (5)
E_2 + A23187	2.12 ± 0.22* (5)
E_2 + nifedipine	1.09 ± 0.28 (6)
E_2 + EGTA	1.05 ± 0.20 (6)
E_2 + BAPTA-AM	0.77 ± 0.12 (6)
E_2BSA	1.70 ± 0.38* (7)
E_2 + tamoxifen	1.98 ± 0.44* (5)
E_2 + ICI 182,780	0.91 ± 0.15 (5)

Note. Values are means ± SD, with the number of measurements shown in parentheses, and represent fold increases as compared with control (NO production without E_2). The absolute amount of NO produced in control is 7.87 ± 1.15 nmol/10^6 cells.

* $p < 0.05$ (vs. control).

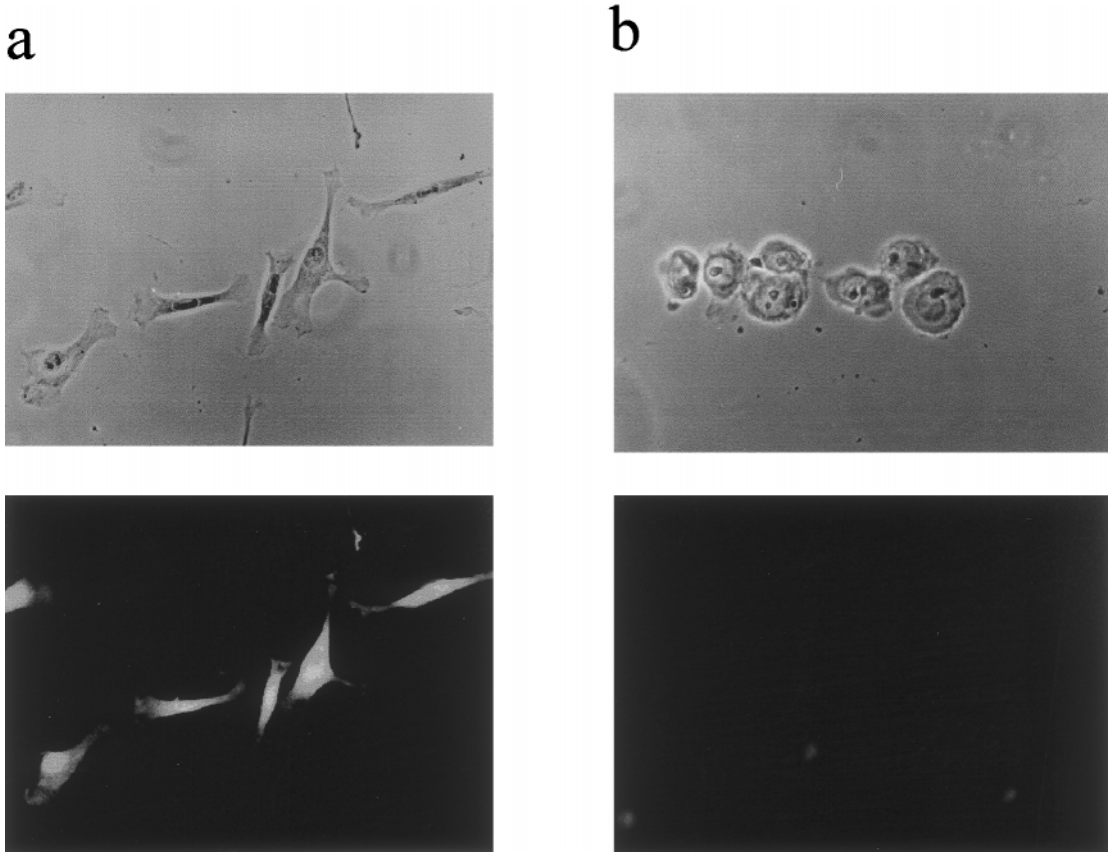

FIG. 2. Binding of E_2BSA-FITC to membranes of (a) BAECs and (b) SK Hep1 cells. Cells were grown on polylysine-coated coverslips and fixed with 4% paraformaldehyde. Fixed cells were incubated with E_2BSA-FITC (20 μM) for 40 min at 4°C after blocking with 2% BSA. BSA-FITC was used in a corresponding control (not shown). The localization of E_2BSA-FITC was analyzed by Zeiss IM 35 microscope.

DISCUSSION

There is considerable evidence in the literature showing that in several vascular and reproductive cell types, acute E_2 treatment exerts early physiological effects that are too rapid to be mediated by genomic activation. These rapid effects of E_2 are instead more compatible with the activation of signal transduction mechanisms similar to those enacted by peptide hormones after their interaction with membrane receptors. Recently, various reports have revealed the presence of cell-surface receptors for E_2 in ECs and various other cell types such as granulosa cells, endometrial cells, oocytes, spermatozoa, and pituitary cells (26–31). However, to date the exact nature of these cell-surface receptors has not been clarified. This study demonstrated for the first time that in BAECs, acute exposure to E_2 increased NO production through the ERα localized in specific plasma membrane domain caveolae.

The kinetic study showed that E_2-stimulated NO production reached its maximum level at 5 min treatment before falling to near basal levels over the next 30 min (Fig. 1). The rapid onset and then attenuation of the E_2 response, along with the observation that it was not accompanied by increased eNOS protein expression (Fig. 1), suggests that it represents a nongenomic action of E_2 and occurs without a need for eNOS upregulation. As mentioned in the Materials and Methods section, the NO production measured by our assay protocol is solely dependent on eNOS activity. The short duration of NO production, thus, suggests that E_2 acutely activates eNOS and deactivates it soon afterwards. It is not clear which signaling mechanisms mediate this brief response, however, we would speculate that the rise in intracellular Ca^{2+} levels by acute E_2 treatment may account for the rapid activation/deactivation of eNOS. It has been previously reported that eNOS is inhibited by its interaction with caveolin in caveolae and that Ca^{2+}/calmodulin releases caveolin from the caveolin-eNOS complex, therefore permitting eNOS activation (20, 21). If this is the case then one could imagine that E_2 directly activates a putative ER, subsequently stimulating a downstream signaling pathway(s) that recruits cofactors such as Ca^{2+}/calmodulin transiently to the plasmalemmal caveolae where most eNOS is localized. At present, however, it is not known how Ca^{2+}/calmodulin uncouples the

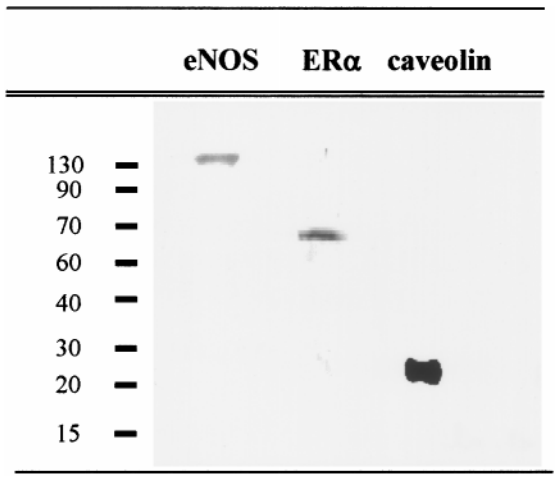

FIG. 3. Identification of eNOS, ERα, and caveolin in caveolae pepared from BAECs. Caveolae were prepared from BAECs as described under Materials and Methods. Caveolar proteins were separated by SDS–polyacrylamide gel electrophoresis and blotted onto nitrocellulose membrane. The blots were immunostained with anti-eNOS, anti-ERα, or anti-caveolin 1 antibodies and developed using enhanced chemiluminescence reagents.

caveolin-eNOS complex in caveolae to activate eNOS and how this activation is terminated. A recent study has shown that 5 min after E_2 treatment, most of the eNOS translocated from the plasma membrane to the intracellular sites close to the nucleus returned back to the plasma membrane on more prolonged E_2 exposure (23). This indicates that intracellular trafficking of eNOS plays an important role in its activity in the cells. Furthermore, in that same study (23), a rapid rise in intracellular Ca^{2+} was observed after only 30 s of E_2 treatment, which returned towards baseline levels within 15 min of hormone treatment. Taken all together, it is likely that the transient intracellular Ca^{2+} mobilization plays an important role in exerting various E_2 responses.

Because raised cytosolic Ca^{2+} levels induce numerous downstream responses, but yet do not mediate acute E_2-induced NO release in HUVECs (12), we reexplored the role of Ca^{2+} in NO release from BAECs in response to acute E_2 treatment. Our data clearly show that both nifedipine and EGTA fully inhibit E_2-stimulated NO production (Table 1), suggesting that the short-term effect of E_2 on NO release from BAECs is dependent on extracellular Ca^{2+}. In addition, A23187 evoked a response which was equivalent in magnitude to the E_2 response, and when BAECs were cotreated with E_2 and A23187 the release of NO was no greater than the effects of the two agents given individually (Table 1). This suggests that E_2-induced NO formation is dependent on cytosolic Ca^{2+}, a conclusion further supported by the fact that BAPTA-AM fully ablated the effect of E_2 (Table 1). The Ca^{2+}-dependence of nongenomic E_2 action reported here is also supported by previous results from other laboratories (23, 24). However, the dependence of the E_2 action on extracellular Ca^{2+} is controversial since acute E_2-induced NO release from chicken granulosa cells was reported to be triggered by Ca^{2+} release into the cytosol from intracellular Ca^{2+} stores (32). The dependence on both extracellular and intracellular Ca^{2+} of the E_2-induced nongenomic effect has also been reported using mouse T cells (33). Different cell culture and experimental conditions may explain these apparently incompatible observations. Furthermore, unlike nifedipine and diltiazem, two other Ca^{2+} channel blockers, namely amlodipine (34) and felodipine (35) were reported to stimulate endothelial NO production rather than inhibit it. This suggests that the role of Ca^{2+} in NO release may be more complicated than is currently thought. In this regard, the Ca^{2+} channel that mediates E_2-stimulated NO production in endothelial cells has yet to be characterized.

Our final goal in this study was to characterize a putative ER existing in the plasma membrane which accounts for the nongenomic E_2 response. In this study the binding of E_2 to the plasma membrane was confirmed by using membrane-impermeable E_2BSA-FITC (Fig. 2) and the localization of ERα in caveolae prepared from ECs was clearly shown by immunoblotting (Fig. 3). The possibility that the classical nuclear receptor ERα exists on the plasma membrane was supported by a recent publication where small numbers of both ERα and ERβ (2–3% of nuclear receptor expression) were expressed in the plasma membrane of Chinese hamster ovary cells transfected with both of the receptors (36). In addition, a pure ERα antagonist ICI 182,780 completely blocked estrogen-stimulated NO release suggesting it is mediated by ERα. In contrast, a partial antagonist tamoxifen did not block this response at all. During the preparation of this manuscript, it has been reported that ERα mediates the nongenomic activation of eNOS by E_2 (24). The results reported from this study (24) are largely compatible with the data from the current study except that tamoxifen treatment was reported to fully inhibit E_2-stimulated NO release contrary to the current data (Table 1). It is not clear whether this discrepancy results from use of different types of cells since in the other study lamb pulmonary artery ECs were used whereas here we used BAECs. More detailed experiments would be necessary to resolve this issue, however, it is interesting to note that tamoxifen serves as an ER antagonist in breast tissue (37) but as an ER agonist in bone (38) and uterine tissue (39). Therefore tissue-specific differences in response to E_2 could account for the differences in the data reported.

It is now believed that increased NO production in response to acute E_2 treatment is mediated by a mechanism independent of the classical genomic pathway of steroid action. Many attempts have been made to iden-

tify the membrane ERs that exert this nongenomic action (28, 40–42) and this aspect of estrogen action remains to be fully understood. The present observations reconfirm the very recent publication (24) that ERα mediates the nongenomic action of E_2. Furthermore, the current study shows that ERα exists in plasmalemmal caveolae. Further studies of the interaction of ERα with other functionally important proteins in caveolae will enhance our current understanding of both the specific role of acute estrogen treatment in the vasculature and perhaps of the general mechanisms of nongenomic actions of other steroid hormone receptors.

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