Estrogen Induces the Akt-dependent Activation of Endothelial Nitric-oxide Synthase in Vascular Endothelial Cells*

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Although estrogen is known to activate endothelial nitric oxide synthase (eNOS) in the vascular endothelium, the molecular mechanism responsible for this effect remains to be elucidated. In studies of both human umbilical vein endothelial cells (HUVECs) and simian virus 40-transformed rat lung vascular endothelial cells (TRLECs), 17β-estradiol (E2), but not 17α-E2, caused acute activation of eNOS that was unaffected by actinomycin D and was specifically blocked by the pure estrogen receptor antagonist ICI-182,780. Treatment of both TRLECs and HUVECs with 17β-E2 stimulated the activation of Akt, and the PI3K inhibitor wortmannin blocked the 17β-E2-induced activation of Akt. 17β-E2-induced Akt activation was also inhibited by ICI-182,780, but not by actinomycin D. Either treatment with wortmannin or exogenous expression of a dominant negative Akt in TRLECs decreased the 17β-E2-induced eNOS activation. Moreover, 17β-E2-induced Akt activation actually enhances the phosphorylation of eNOS. 17β-E2-induced Akt activation was dependent on both extracellular and intracellular Ca2+. We further examined the 17β-E2-induced Akt activity in Chinese hamster ovary (CHO) cells transiently transfected with cDNAs for estrogen receptor α (ERα) or estrogen receptor β (ERβ). 17β-E2 stimulated the activation of Akt in CHO cells expressing ERα but not in CHO cells expressing ERβ. Our findings suggest that 17β-E2 induced eNOS activation through an Akt-dependent mechanism, which is mediated by ERα via a nongenomic mechanism.

The inhibitory effect of estrogen on the development of atherosclerosis has been suggested by abundant human epidemiologic and animal experimental data (1–9). The incidence of atherosclerotic diseases is lower in premenopausal women than in men, steeply rises in postmenopausal women, and is reduced to postmenopausal levels in postmenopausal women who receive estrogen therapy (10–12). Until recently, the atheroprotective effects of estrogen were attributed principally to the effects on serum lipid concentrations. However, estrogen-induced alterations in serum lipids account for only approximately one-third of the observed clinical benefits of estrogen (12–14). Recent evidence suggests that the direct actions of estrogen on blood vessels contribute to the cardioprotective effects of estrogen (13, 15). There are many kinds of direct effects of estrogen on blood vessels, such as estrogen-induced increases of vasodilatation and inhibition of the response of blood vessels to injury and the development of atherosclerosis. However, the molecular mechanism underlying the estrogen-induced vasodilatation has not yet been determined. Several studies suggest that a key mediator of this vasodilator response could be the endothelium-derived relaxing factor nitric oxide (NO), and that brief treatment with estrogen increases basal NO release in endothelial cells without elevation of eNOS mRNA or protein (16). Estrogen activates endothelial nitric oxide synthase (eNOS) without altering expression of the eNOS gene in vascular endothelium (17–20). However, the details of the mechanism of the estrogen-induced eNOS activation are not yet well understood.

The serine/threonine kinase termed Akt or protein kinase B (PKB) is an important regulator of various cellular processes, including glucose metabolism and cell survival (21, 22). Activation of receptor tyrosine kinases and G-protein-coupled receptors, and stimulation of cells by mechanical force, can lead to the phosphorylation and activation of Akt (23–25). Akt was identified as a downstream component of survival signaling through phosphatidylinositol 3-kinase (PI3K) (26–30). Akt may be regulated by both phosphorylation and the direct binding of PI3K lipid products to the Akt pleckstrin homology domain. Akt can then phosphorylate substrates such as glycogen synthase kinase-3, 6-phosphofructo-2-kinase, and BAD. More recently, it was found that eNOS is also an Akt substrate and is activated by Akt-dependent phosphorylation to release NO in endothelial cells (31–34).

The actions of estrogen can be mediated by the classical nuclear receptors, ERα and ERβ (35, 36) or through other putative membrane receptors. By definition, rapid effects of estrogen that involve nongenomic mechanisms are independent of transcriptional activation by the nuclear ERs. These rapid effects are believed to be mediated by receptors located in or close to the plasma membrane (37, 38). Estrogen-induced vasodilatation occurs 5–20 min after estrogen administration (39, 40) and is not dependent on changes in gene expression; therefore, the rapid effects of estrogen may occur through nongenomic mechanisms.

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this action of estrogen is sometimes referred to as “non-genomic.” Therefore, we sought to determine whether the estrogen-induced eNOS activation is mediated by Akt activation and which type of ER is involved in this effect using both human umbilical vein endothelial cells (HUVECs) and simian virus 40-transformed rat lung vascular endothelial cells (TRLECs) (41).

EXPERIMENTAL PROCEDURES

Materials—17β-E2, 17α-E2, E2–17-BSA (17-hemisuccinyl/BSA; 38 mol E2/mol BSA), actinomycin D, and wortmannin were purchased from Sigma Chemical Co. (St. Louis, MO). ICI-182,780 was obtained from Tocris (Ballwin, MO). ECL Western blotting detection reagents were obtained from Amersham Pharmacia Biotech (Arlington Heights, IL). Rabbit polyclonal anti-Akt antibody and an Akt kinase assay kit, including GSK-3 fusion protein and a phospho-specific GSK-3α/β antibody, were obtained from New England BioLabs (Beverly, MA). Rabbit polyclonal anti-hemagglutinin (HA) antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell Cultures—TRLECs (41), kindly provided by Dr. K. Fukuo and Dr. S. Morimoto (Osaka University Medical School, Japan), and Chinese hamster ovary (CHO) cells, obtained from American Type Culture Collection (Rockville, MD), were cultured at 37 °C in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum in a water-saturated atmosphere of 95% O2 and 5% CO2. HUVECs were isolated according to the method of Jaffe et al. (42), plated in gelatin-coated tissue culture wells, and grown in M199 medium containing 20% fetal bovine serum and 50 μg/ml endothelial cell growth supplement (Clonetics Corp., San Diego, CA). HUVECs were used at passage 2 or 3.

Constructs—The vectors encoding the various HA-tagged forms of Akt, wild-type Akt (HA-Akt), kinase-inactive Akt (HA-AktK179M), and constitutively active Akt (HA-mD4–129Akt) used in this study have been described previously (26, 43, 45–47). The vectors encoding the wild-type eNOS and mutant eNOS of serine 1179 to alanine (S1179A eNOS) was a kind gift from Dr. W. C. Sessa (Yale University, New Haven, CT) (31). The human estrogen receptor α (ERα) expression vector, pSG5-HEGO, was a kind gift from Dr. P. Chambon (Institut de Chimie Biologique, Strasbourg, France) (48). The plasmid pSG5-mERβ encoding nucleotides 12–1469 of ERβ (35) was kindly provided Dr. E. R. Levin (University of California, Irvine, CA) via Dr. K. S. Korach (National Institutes of Health, Research Triangle Park, NC).

Assay of eNOS Activity—Cells were serum-starved overnight in phenol red-free medium before eNOS activity measurements. eNOS activity was determined as the conversion of radiolabeled L-arginine to L-citrulline, as described under “Experimental Procedures.” The basal activity of eNOS was arbitrarily set at 1.0. Data are expressed as the mean ± S.E. of six separate experiments. *p < 0.05 and **p < 0.01 as compared with the control, respectively.

![Fig. 1. Activation of eNOS in endothelial cells.](http://www.jbc.org/)

**A** TRLEC

- **control**
- **17βE2: 10^{-7}M**

**B** HUVEC

- **control**
- **17βE2: 10^{-7}M**

Experimental procedures were as follows:

- **Materials:**
  - 17β-E2, 17α-E2, E2–17-BSA.
  - Actinomycin D, and wortmannin.
  - ICI-182,780.
  - ECL Western blotting detection reagents.
  - Rabbit polyclonal anti-Akt antibody.
  - Akt kinase assay kit.
  - Rabbit polyclonal anti-hemagglutinin (HA) antibody.
- **Cell Cultures:**
  - TRLECs.
  - Chinese hamster ovary (CHO) cells.
  - HUVECs.
- **Constructs:**
  - HA-Akt, HA-AktK179M, HA-mD4–129Akt.
  - Wild-type eNOS and mutant eNOS.
  - ERα expression vector.
- **Assay of eNOS Activity:**
  - Used M199 medium.
  - Assayed L-arginine to L-citrulline.

**Fig. 1**

- **A** TRLEC
  - **control**
  - **17βE2: 10^{-7}M**

- **B** HUVEC
  - **control**
  - **17βE2: 10^{-7}M**

- **Basal activity of eNOS** was set at 1.0.
- **Data** expressed as mean ± S.E.
- **p < 0.05** and **p < 0.01** compared with control.
buffer A. Citrulline was eluted with 0.5 ml of buffer B, and then radioactivity was measured with a liquid scintillation counter.

**Assay of eNOS Activity Using a Transient Expression System**—TRLECs cultured in 100-mm dishes were transfected with 1 µg of CMV-6, 1 µg of CMV-6 containing the gene for HA-AktK179M, 1 µg of pSG5, 1 µg of Erk expression vector (pSG5-HEGO), or 1 µg of ERβ expression vector (pSG5-mERβ) using LipofectAMINE plus (Life Technologies, Inc.) as described previously (52, 53). Seventy-two hours after transfection, serum-deprived cells were incubated with 10⁻⁷ M 17β-E2 for 15 min, and the eNOS activity was measured as described above.

**Assay of Akt Kinase Activity**—Cells were serum-starved overnight in phenol red-free medium and then treated with various materials. They were then washed twice with phosphate-buffered saline and lysed in ice-cold lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). The extracts were centrifuged to remove cellular debris, and the protein content of the supernatants was determined using the Bio-Rad protein assay reagent. 250 µg of protein from the cell lysates was subjected to immunoprecipitation with anti-Akt antibody. For assay using a transient expression system, TRLECs cultured in 100-mm dishes were transfected with 1 µg of pSG5, 1 µg of Erk expression vector (pSG5-HEGO), or 1 µg of ERβ expression vector (pSG5-mERβ) using LipofectAMINE plus as described previously (52, 53). Seventy-two hours after transfection, serum-deprived cells were incubated with 10⁻⁷ M 17β-E2 for 15 min, and the Akt activity was measured as described above.

**Preparation of Partially Purified eNOS**—Human eNOS was overexpressed in SF-21 cells, which had been infected with baculovirus carrying human eNOS cDNA (56). Human eNOS was partially purified by chromatography on 2',5'-ADP-Sepharose gel, and its specificity was determined as described previously (57).

**Assay of eNOS Phosphorylation**—TRLECs cultured in 100-mm dishes were treated with 10⁻⁷ M 17β-E2 for 15 min. Cell lysates were subjected to immunoprecipitation with anti-Akt antibody. For assay using a transient expression system, TRLECs cultured in 100-mm dishes were transfected with 1 µg of HA-Akt, 1 µg of HA-AktK179M, or 1 µg of HA-mΔ4–129Akt using LipofectAMINE plus (Life Technologies, Inc.) as described previously (52, 53). Seventy-two hours after transfection, serum-deprived cells were incubated with 10⁻⁷ M 17β-E2 for 15 min, and lysates were immunoprecipitated with anti-HA antibody. The immunoprecipitated products were washed once in lysis buffer and twice in kinase assay buffer, and samples were resuspended in 30 µl of kinase assay buffer containing 40 µCi [γ-³²P]ATP (1 µCi) and 5 µg of partially purified eNOS, or 5 µg of recombinant wild-type or S1179A eNOS purified from Escherichia coli. The kinase reaction was allowed products were resolved by 5% SDS-PAGE followed by Western blotting (55) with an anti-phospho-GSK-3β antibody as described previously (47).

**Assay of Akt Activity Using a Transient Expression System**—CHO cells cultured in 100-mm dishes were transfected with 1 µg of pSG5, 1 µg of Erk expression vector (pSG5-HEGO), or 1 µg of Erk expression vector (pSG5-mERβ) using LipofectAMINE plus as described previously (52, 53). Seventy-two hours after transfection, serum-deprived cells were incubated with 10⁻⁷ M 17β-E2 for 15 min, and the Akt activity was measured as described above.

**Preparation of Partially Purified eNOS**—Human eNOS was overexpressed in SF-21 cells, which had been infected with baculovirus carrying human eNOS cDNA (56). Human eNOS was partially purified by chromatography on 2',5'-ADP-Sepharose gel, and its specificity was determined as described previously (57).

**Assay of eNOS Phosphorylation**—TRLECs cultured in 100-mm dishes were treated with 10⁻⁷ M 17β-E2 for 15 min. Cell lysates were subjected to immunoprecipitation with anti-Akt antibody. For assay using a transient expression system, TRLECs cultured in 100-mm dishes were transfected with 1 µg of HA-Akt, 1 µg of HA-AktK179M, or 1 µg of HA-mΔ4–129Akt using LipofectAMINE plus (Life Technologies, Inc.) as described previously (52, 53). Seventy-two hours after transfection, serum-deprived cells were incubated with 10⁻⁷ M 17β-E2 for 15 min, and lysates were immunoprecipitated with anti-HA antibody. The immunoprecipitated products were washed once in lysis buffer and twice in kinase assay buffer, and samples were resuspended in 30 µl of kinase assay buffer containing 40 µCi [γ-³²P]ATP (1 µCi) and 5 µg of partially purified eNOS, or 5 µg of recombinant wild-type or S1179A eNOS purified from Escherichia coli. The kinase reaction was allowed to proceed at 30 °C for 30 min and stopped by the addition of Laemmli SDS sample buffer (54). Reaction
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RESULTS

eNOS Activation by 17β-E2—To evaluate whether eNOS is activated by 17β-E2 in TRLECs (Fig. 1A, upper panel) and HUVECs (Fig. 1A, lower panel), cultured cells were exposed to 17β-E2 for the indicated times. The increase in eNOS activity induced by $10^{-7} \text{M} \ 17β-E2$ reached a plateau from 15 through 30 min and rapidly declined thereafter. The dose dependence of 17β-E2-induced eNOS activation was also evaluated in TRLECs (Fig. 1B). TRLECs were treated with various concentrations of 17β-E2 for 15 min. In the range of $10^{-10}$ to $10^{-7} \text{M}$, 17β-E2 induced the activation of eNOS in a dose-dependent manner. A higher concentration ($10^{-6} \text{M}$) of 17β-E2 did not induce a stronger response (data not shown). The response was specific for 17β-E2, because 17α-E2 had no effect (Fig. 2A). To determine whether this response involves rapid ER activation, the effect of concomitant treatment with the pure ER antagonist ICI-182,780 was determined (Fig. 2B). ICI-182,780 completely abolished the induction of eNOS activation by 17β-E2. Moreover, the effects of E2–17-BSA, a membrane-impermeable conjugate of E2, and actinomycin D, an inhibitor of gene transcription, were tested to rule out the influence of genomic effects mediated by nuclear ERs (Fig. 2C). E2–17-BSA stimulated an increase in eNOS activity similar to that induced by 17β-E2, and actinomycin D did not affect the induction of eNOS activation by 17β-E2.

Activation of Akt by 17β-E2—To determine whether Akt is activated by 17β-E2 in TRLECs and HUVECs, 17β-E2 was added to cultured cells for the indicated times (Fig. 3A) and at the indicated concentrations for 15 min (Fig. 3B). Cell lysates were subjected to immunoprecipitation with immobilized anti-Akt antibody, and then supplemented with GSK-3α/β fusion protein and analyzed by Western blotting with anti-phospho-GSK-3α antibody. Activation of Akt by 17β-E2 in both TRLECs and HUVECs reached a plateau at 15 min, and declined thereafter (Fig. 3A). 17β-E2 induced the activation of Akt in a dose-dependent manner in TRLECs (Fig. 3B) and HUVECs (data not shown). The response was specific for 17β-E2, because $10^{-10}$–$10^{-7} \text{M} \ 17α-E2$ had no effect (Fig. 4A). Because Akt is an effector of survival signaling downstream of PI3K (26–30), we next examined whether stimulation of TRLECs with 17β-E2 could increase the activity of Akt through a PI3K-deendent mechanism. TRLECs were stimulated with 17β-E2 in the presence or absence of wortmannin, a PI3K inhibitor, and the kinase activity of Akt was assayed. The induction of Akt

![Fig. 3](image-url)

**Fig. 3. Activation of Akt by 17β-E2 in endothelial cells.** Cells were grown in 100-mm dishes. A, TRLECs (upper panel) and HUVECs (lower panel) were treated with $10^{-7} \text{M} \ 17β-E2$ for the indicated times. B, TRLECs were treated with the indicated concentrations of 17β-E2 for 15 min. Lysates were subsequently subjected to immunoprecipitation with immobilized anti-Akt antibody, and the kinase reaction was carried out in the presence of cold ATP and GSK-3α fusion protein, as described under “Experimental Procedures.” After the reactions were stopped with Laemmli sample buffer, samples were resolved by 12% SDS-PAGE and then analyzed by Western blotting with an anti-phospho-GSK-3α/β antibody. Experiments were repeated three times with essentially identical results.

![Fig. 4](image-url)

**Fig. 4. Specificity of the augmentation of Akt activation by 17β-E2.** Cells were grown in 100-mm dishes. A, TRLECs were treated with $10^{-7} \text{M} \ 17β-E2$ or the indicated concentrations of 17α-E2 for 15 min. B, TRLECs were pretreated with or without $2 \times 10^{-7} \text{M} \ \text{wortmannin}$ for 15 min and followed by treatment with $10^{-7} \text{M} \ 17β-E2$ for 15 min. C, TRLECs were pretreated with or without 25 μg/ml actinomycin D (Act-D) for 120 min, followed by treatment with $10^{-7} \text{M} \ 17β-E2$ or $10^{-7} \text{M} \ 17α-E2$–17-BSA for 15 min. Experiments were repeated three times with essentially identical results.
activity by 17β-E2 was inhibited by wortmannin (Fig. 4B, lane 6). These results indicate that E2 activates Akt activity through a PI3K-dependent mechanism.

To determine whether this process involves rapid ER activation, the effect of concomitant treatment with the pure ER antagonist ICI-182,780 was determined (Fig. 4B, lane 4). ICI-182,780 clearly caused a decrease in 17β-E2-induced Akt activation. Moreover, E2–17-BSA, a membrane-impermeable conjugate of E2, and actinomycin D, an inhibitor of gene transcription, were used to rule out the influence of genomic events mediated by nuclear ERs (Fig. 4C and D). E2–17-BSA also stimulated an increase in Akt activity, and actinomycin D did not affect the induction of Akt activity by 17β-E2.

Akt-dependent eNOS Phosphorylation and Activation—To determine whether 17β-E2-induced Akt activation is involved in the phosphorylation of eNOS, 10⁻⁷ m 17β-E2 was added to cultured cells for 15 min. Cell lysates were subjected to immunoprecipitation with anti-Akt antibody, and then assayed in an immunocomplex kinase assay using purified eNOS (57) as a substrate (Fig. 5A, left panel). 17β-E2 directly increased the phosphorylation of eNOS in anti-Akt immunoprecipitates. Moreover, we evaluated the effect of exogenous expression of various forms of Akt on the in vitro phosphorylation of purified eNOS. TRLEC transfected with wild-type or mutant forms of hemagglutinin (HA)-tagged Akt were exposed to 10⁻⁷ m 17β-E2 for 15 min, and extracts from these cells were immunoprecipitated with anti-HA antibody and assayed in an immunocomplex kinase assay for their ability to phosphorylate purified eNOS. Akt constructs that were expressed in TRLEC included HA-tagged wild-type Akt (HA-Akt), an Akt derivative rendered kinase-inactive by point mutation within the Akt catalytic domain (HA-AktK179M), and an Akt derivative rendered constitutively active by targeting it to the plasma membrane with a myristoyl tag (HA-m Musk–K179M) (26, 43, 45–47). 17β-E2 directly increased the phosphorylation of eNOS in anti-HA immunoprecipitates prepared from TRLEC transfected with wild-type Akt (Fig. 5A, lane 2). Anti-HA immunoprecipitates prepared from TRLEC transfected with the kinase-inactive Akt failed to phosphorylate eNOS induced by 17β-E2 (Fig. 5A, lane 4). In addition, anti-HA immunoprecipitates from TRLEC
transfected with constitutively active Akt were found to induce eNOS phosphorylation in immunocomplex kinase assays (Fig. 5A, lane 5). Because it was reported that Akt directly phosphorylated on serine 1179 (31), an immunocomplex kinase assay with anti-Akt antibody was performed using recombinant wild-type eNOS or mutant eNOS of serine 1179 to alanine (eNOS S1179A) as a substrate (Fig. 5B). Mutation of serine 1179 to alanine markedly reduced 17β-E2-induced phosphorylation of eNOS compared with the wild-type protein. These results suggest that 17β-E2-induced Akt activation actually increases the phosphorylation of eNOS. Next, we sought to determine whether an Akt cascade is involved in the regulation of the eNOS activation induced by 17β-E2 in the endothelial cells. To examine whether the stimulation of the eNOS activation by 17β-E2 is the result of activation of Akt, either wortmannin (Fig. 5C) or an expression vector, kinase-inactive HA-AktK179M, was used (Fig. 5D). Pretreatment with 2 × 10⁻⁷ M wortmannin for 15 min completely abolished the 17β-E2-induced eNOS activation (Fig. 5C). In addition, transfection with HA-AktK179M clearly abolished the 17β-E2-induced eNOS activation, whereas transfection with control vector had no effect on the 17β-E2-induced eNOS activation (Fig. 5D). These results suggest that the PI3K-Akt cascade is involved in the 17β-E2-induced eNOS activation.

Role of Extracellular and Intracellular Ca²⁺ in 17β-E2-induced Akt and eNOS Activation—eNOS is a Ca²⁺/calmodulin-dependent enzyme, and it has been reported that estrogen induces translocation of eNOS in a Ca²⁺-dependent and receptor-mediated manner (58). A23187 induces eNOS activation and produces endothelium-dependent vascular relaxation (19, 59). Thus, eNOS activity is largely regulated by Ca²⁺ mobilization. We therefore evaluated the role of extracellular and intracellular Ca²⁺ in 17β-E2-induced Akt and eNOS activation in TRLECs (Fig. 6). Elimination of extracellular Ca²⁺ by treatment with 3 mM EGTA for 1 min clearly blocked the A23187-induced Akt (Fig. 6A, upper panel) and eNOS (Fig. 6A, lower panel) activation, and similarly, treatment with 3 mM EGTA for 1 min clearly inhibited the 17β-E2-induced Akt (Fig. 6A, upper panel) and eNOS (Fig. 6A, lower panel) activation, indicating that Ca²⁺ influx is required for 17β-E2-induced Akt and eNOS activation. Next, the effect of intracellular Ca²⁺ on 17β-E2-induced Akt and eNOS activation was examined (Fig. 6B). Treatment with 50 μM 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic-acetoxymethyl ester (BAPTA-AM) for 20 min to eliminate intracellular Ca²⁺ (52, 53) completely blocked the 17β-E2-induced Akt (Fig. 6B, upper panel) and eNOS (Fig. 6B, lower panel) activation. Moreover, elimination of both extracellular and intracellular Ca²⁺ by treatment with 3 mM EGTA for 15 min (52, 53, 60) abolished the 17β-E2-induced Akt (Fig. 6B, upper panel) and eNOS (Fig. 6B, lower panel) activation, indicating that intracellular Ca²⁺ is also required for 17β-E2-induced Akt and eNOS activation. Thus, these results suggest that Ca²⁺ mobilization mediated by both extracellular and intracellular Ca²⁺ is required for the 17β-E2-induced Akt and eNOS activation.

Effect of ERα or ERβ Expression on 17β-E2-induced Akt and eNOS Activation—The potential role of ERα or ERβ in 17β-E2-induced Akt activation was evaluated. Transfection of ERβ into TRLECs had no effect on 17β-E2-induced Akt activation compared with transfection of control vector (Fig. 7A, upper panel). On the other hand, transfection of ERα into TRLECs caused an increase in both basal and 17β-E2-induced Akt activation compared with transfection of control vector (Fig. 7A, upper panel). Moreover, transfection of ERα into TRLECs caused an increase
**Fig. 7. Effect of ERα or ERβ expression on 17β-E2-induced Akt and eNOS activation.** Cells were grown in 60-mm dishes. A, TRLECs were transfected with control vector (pSG5), ERα expression vector (pSG5-HEGO), or ERβ expression vector (pSG5-mERβ) and, after 72 h, were stimulated with 10⁻⁷ M 17β-E2 for 15 min. Akt activity (upper panel) and eNOS activity (lower panel) were measured as described in the legend for Figs. 3 and 1, respectively. The basal activity of eNOS of transfected cells was arbitrarily set at 1.0. Data are expressed as the mean ± S.E. of six separate experiments. **p < 0.01 as compared with the control. B, CHO cells were transfected with empty vector (pSG5), ERα expression vector (pSG5-HEGO), or ERβ expression vector (pSG5-mERβ) and, after 72 h, were stimulated with 10⁻⁷ M 17β-E2 for 15 min. Akt activity was measured as described in the legend for Fig. 3. Experiments were repeated three times with essentially identical results.

in 17β-E2-induced eNOS activation compared with transfection of control vector or of ERβ (Fig. 7A, lower panel). We confirmed that both ERα and ERβ were expressed in TRLECs (data not shown). Therefore, CHO cells, which do not express ERα or ERβ (61), were used to examine which of these receptors is involved in 17β-E2-induced Akt activation. In CHO cells transfected with control vector or ERβ, 17β-E2 had no effect on Akt activity (Fig. 7B). However, in cells transfected with ERα, there was an apparent increase in Akt activity upon brief stimulation with 17β-E2 (Fig. 7B). These results indicate that 17β-E2 induces Akt activation through ERα.

**DISCUSSION**

This study showed that Akt is activated in HUVECs and TRLECs by 17β-E2 and that Akt activation is required for the activation of eNOS following brief treatment with 17β-E2: Treatment with wortmannin, a PI3K inhibitor, attenuated the 17β-E2-induced Akt and eNOS activation, and TRLECs expressing inactive Akt showed less induction by 17β-E2 of eNOS activity. A pure ER antagonist, ICI-182,780 completely inhibited the 17β-E2-induced Akt and eNOS activation. Moreover, in CHO cells transfected with ERα, there was an apparent increase in Akt activity upon brief stimulation with 17β-E2. These results suggest that a 17β-E2-induced PI3K-Akt cascade stimulates the activation of eNOS through ERα.

Normal endothelium secretes nitric oxide, which relaxes vascular smooth muscle and inhibits platelet activation (62). In cultured endothelial cells, physiologic concentrations of estrogen cause a rapid release of nitric oxide without altering gene expression (17, 18). The rapidity of the activation of Akt (Fig. 3) and eNOS (Fig. 1) by 17β-E2 along with the fact that the activation was not altered by the inhibition of gene transcription by actinomycin D indicate that the process may not require the classical nuclear effects of the estrogen. However, the acute response of Akt (Fig. 4B) and eNOS (Fig. 2B) to 17β-E2 was fully inhibited by coconcurrent treatment with the pure ER antagonist ICI-182,780, suggesting that the response requires a rapid ER activation. Are these rapid effects of estrogen mediated by an unidentified estrogen receptor or by the known estrogen receptors acting in a novel way? The existence of rapidly acting membrane receptors for steroid hormones in both nonvascular and vascular cells has been suggested for over two decades (63, 64), but no such receptors have been isolated or cloned. Alternatively, the rapid effects of estrogen on vascular cells could be mediated by a known estrogen receptor, perhaps located in the plasma membrane (64), which is able to activate nitric oxide synthase in a nongenomic manner (19, 20). In addition, estrogen increases the expression of genes for important vasodilatory enzymes such as prostacyclin synthase and nitric oxide synthase (65, 66). Some of the effects of estrogen may therefore be due to longer-term increases in the expression of the genes for these enzymes in vascular tissues.

There are two estrogen receptors, estrogen receptor α (ERα) and estrogen receptor β (ER β), both of which are members of the superfamily of steroid hormone receptors (35, 67). Genetic disruption of ERα in mice leads to lower levels of vascular nitric oxide (69). In addition, ERα can directly activate endothelial nitric oxide synthase (19, 20). We found that there was an apparent increase in Akt activity upon brief stimulation with
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17β-E2 in CHO cells transfected with ERα, whereas 17β-E2 had no effect on Akt activity in cells transfected with control vector or ERβ (Fig. 7B). These findings suggest that ERα is capable of mediating the acute response and rapid vasodilatation caused by estrogen. What is the role of ER β in endothelial cells? In addition to forming homodimers, ERα and ERβ can form heterodimers with each other (70), adding a further degree of complexity in the regulation of gene expression by estrogen in cells expressing both receptors. Estrogen continues to provide protection against vascular injury in mice in which ERα has been disrupted (71), and the expression of ERβ, but not ERα, is elevated after vascular injury in male rats (72). Estrogen also provides protection against vascular injury in mice in which ERβ has been disrupted (73), suggesting the possibility that either of the two known estrogen receptors is sufficient to protect against vascular injury or that some other unknown signaling pathway is involved.

eNOS is a Ca2+/calmodulin-dependent enzyme, and it has been reported that estrogen induces translocation of eNOS by a Ca2+/calmodulin-dependent receptor-activated pathway (58). Because C2A interacts with and activates eNOS via a novel Ca2+/calmodulin-dependent protein kinase can activate and phosphorylate Akt (74). In addition, A23187 also activates Akt (75). More recently, it was also reported that plasma-lamellar caveolae-associated ERα mediates the acute estrogen-stimulated NO production which occurs via a novel Ca2+/calmodulin-dependent signaling pathway (20). We confirmed that 17β-E2-induced eNOS activation was dependent on both extracellular and intracellular Ca2+ (Fig. 6, lower panel). Interestingly, although it was reported that phosphorylation of eNOS by Akt represents a novel Ca2+-independent regulatory mechanism for activation of eNOS (31–34, 76), the current findings indicate that 17β-E2-induced Akt activation was dependent on both extracellular and intracellular Ca2+ (Fig. 6, upper panel).

Estrogen can cause the rapid activation of signaling pathways involving c-src-related tyrosine kinases and MAP kinases in nonendothelial cells (77, 78). It has also been reported that tyrosine kinase-MAP kinase signaling is involved in acute ERα-mediated eNOS activation in endothelial cells (19, 79). Although no direct evidence that eNOS is one of the substrates of MAP kinase has been reported, it has been reported very recently that eNOS is a substrate of Akt in endothelial cells (31–34), and that PI3K and Akt contribute to the production of NO stimulated by insulin in endothelial cells (80). In addition, estrogen stimulates the activation of Akt, and Akt is a downstream effector of estrogen-dependent proliferation and survival in hormone-responsive MCF-7 breast carcinoma cells (81). However, until recently there had not been any studies addressing the role of the PI3K-Akt cascade in estrogen-induced eNOS activation. This is the first report showing that estrogen stimulates the activation of the PI3K-Akt cascade in endothelial cells and that this cascade might be involved in the eNOS activation induced by estrogen. Is there any cross-talk between the MAP kinase and PI3K-Akt signaling cascades? Although we and other groups (44, 49, 68) reported that MAP kinase and PI3K-Akt signaling cascades converge at BAD to suppress the apoptotic effect of BAD, further investigations are necessary to examine whether MAP kinase and PI3K-Akt signaling cascades converge at eNOS to stimulate the release of NO. We are currently investigating this possibility.

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