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

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ABSTRACT

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.

INTRODUCTION

Materials

| 17β-E2, 17α-E2, E2–17–BSA (17β-estradiol (17-hemisuccinate/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 Toecirs (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 GS-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 accord
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 ERα 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-Akt179M, or 1 μg of HA-mA4–129Akt 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 μM [γ-32P]ATP (1 μCi) and 5 μg of partially purified eNOS, or 5 μg of recombinant wild-type or SI179A eNOS purified from Escherichia coli. The kinase reaction was allowed to proceed at room temperature for 5 min and stopped by the addition of Laemmli SDS sample buffer (54). Reaction products were resolved by 8% SDS-PAGE.

Statistics

Statistical analysis was performed using Student’s t test, and p < 0.05 was considered significant. Data are expressed as the mean ± S.E.

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 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 M, 17β-E2 induced the activation of eNOS in a dose-dependent manner. A higher concentration (10−6 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 events 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 fusion protein and analyzed by Western blotting with anti-phospho-GSK-3α/β antibody. Activation of Akt by 17β-E2 in both TRLECs and HUVEC...
Figure 1: Activation of eNOS in endothelial cells. Cells were grown in 60-mm dishes. A, TRLECs [upper panel] and HUVECs [lower panel] were treated with 10−7 M 17β-E2 for the indicated times. B, TRLECs were treated with the indicated concentrations of 17β-E2 for 15 min. eNOS activity was measured by the conversion of L- [guanidino-14C]arginine to [guanidino-14C]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.

VECs reached a plateau at 15 min, and declined thereafter (Fig. 3 A). 17β-E2 induced the activation of Akt in a dose-dependent manner in TRLECs (Fig. 3 B) and HUVECs (data not shown). The response was specific for 17β-E2, because 10−10–10−7 M 17α-E2 had no effect (Fig. 4 A). 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-dependent 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 activity by 17β-E2 was inhibited by wortmannin (Fig. 4 B, lane 6). These results indicate that E2 activates Akt activity through a PI3K-dependent mechanism.

Figure 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 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/β antibody, 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.

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. 4 A, 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-mΔ4–129Akt) (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. 5, A, lane 2). Anti-HA immunoprecipitates prepared from TRLEC transfected with the kinase-inactive Akt failed to phosphorylate eNOS induced by 17β-E2 (Fig. 5, A, 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. 5, A, 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. 5 B). 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. 5 C) or an expression vector, kinase-inactive HA-AktK179M, was used (Fig. 5 D). Pretreatment with 2 × 10−7 M wortmannin for 15 min completely abolished the 17β-E2-induced eNOS activation (Fig. 5 C). 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. 5 D). These results suggest that the PI3K-Akt cascade is involved in the 17β-E2-induced eNOS activation.

Figure 5: Akt-dependent eNOS phosphorylation and activation. A, the effect of expressed Akt on the phosphorylation of purified eNOS induced by 10−7 M 17β-E2 for 15 min was examined. Immunocomplex kinase assays were performed using anti-Akt immunoprecipitates from TRLECs (left panel) or using anti-HA immunoprecipitates from TRLECs expressing HA-tagged Akt constructs encoding HA-Akt (Wild-type Akt), kinase-inactive HA-AktK179M (Inactive Akt), or constitutively active HA-md4–129 Akt (Active Akt) expressed in TRLECs (right panel). B, the effect of 17β-E2 on the phosphorylation of recombinant wild-type eNOS or eNOS S1179A was examined. Immunocomplex kinase assays were performed using anti-Akt immunoprecipitates from TRLECs treated with or without 10−7 M 17β-E2 for 15 min. C and D, the effect of wortmannin and kinase-deficient Akt on 17β-E2-induced eNOS activation was examined. TRLECs were grown in 60-mm dishes. Cells were pretreated with or without 2 × 10−7 M wortmannin for 15 min, followed by treatment with 10−7 M E2 for 15 min (C) or cells were transfected with control vector (CMV-6) or kinase-inactive HA-AktK179M (Inactive Akt) and, after 72 h, were stimulated with 10−7 M E2 for 15 min (D). eNOS activity was measured as described in the legend for Fig. 1. The basal activity of eNOS of parent cells (C) or cells transfected with CMV-6 (D) 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.

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. 6 A, upper panel) and eNOS (Fig. 6 A, lower panel) activation, and similarly, treatment with 3 mM EGTA for 1 min clearly inhibited the 17β-E2-induced Akt (Fig. 6 A, upper panel) and eNOS (Fig. 6 A, 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. 6 B). Treatment with 50 μM 1,2-bis[α-aminophenoxy]ethane-N,N′,N′-tetraacetic-acetoxymethyl ester (BAPTA-AM) for 20 min to eliminate intracellular Ca2+ (52, 53) completely blocked th e 17β-E2-induced Akt (Fig. 6 B, upper panel) and eNOS (Fig. 6 B, 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. 6 B, upper panel) and eNOS (Fig. 6 B, 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. 7 A, 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. 7 A, upper panel). Moreover, transfection of ERα into TRLECs caused an increase in 17β-E2-induced eNOS activation compared with transfection of control vector or of ERβ (Fig. 7 A, 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 with ERβ, 17β-E2 had no effect on Akt activity (Fig. 7 B). However, in cells transfected with ERα, there was an apparent increase in Akt activity upon brief stimulation with 17β-E2 (Fig. 7 B). These results indicate that 17β-E2 induces Akt activity through ERα.

Figure 6: Role of extracellular and intracellular Ca2+ in 17β-E2-induced Akt and eNOS Activation. A, cells were pretreated with 3 mM EGTA for 1 min, and then treated with 10−6 M A23187 or 10−7 M 17β-E2 for 15 min. B, cells were pretreated with 50 μM BAPTA-AM for 20 min or 3 mM EGTA for 15 min, and then treated with 10−7 M 17β-E2 for 15 min. Akt activity (upper panel) and eNOS activity (lower panel) were measured as described in the legends for Figs. 3 and 1, respectively. The basal activity of eNOS was arbitrarily set at 1.0. Data are expressed as the mean ± S.E. of six separate experiments. **p < 0.01 as compared with the control.

Figure 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.
induces eNOS activation and produces endothelium-dependent and receptor-mediated mechanism (58). Because A23187 injury or that some other unknown signaling pathway is involved. Known estrogen receptors is sufficient to protect against vascular disruption (73), suggesting the possibility that either of the two 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 the genes for important vasodilatory enzymes such as prostacyclin synthase (19,20). In addition, estrogen increases the expression of genes for these enzymes in vascular tissues. There are two estrogen receptors, estrogen receptor α (ERα) and estrogen receptor β (ERβ), 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 (31-34, 76), the current findings indicate that rapid effects of estrogen may therefore be due to longer-term increases in the expression of the genes for these enzymes in vascular tissues.

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