Induction of Endothelial Nitric-oxide Synthase Phosphorylation by the Raloxifene Analog LY117018 Is Differentially Mediated by Akt and Extracellular Signal-regulated Protein Kinase in Vascular Endothelial Cells*

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Koji Hisamoto‡, Masahide Ohmichi‡‡, Yuki Kanda‡, Kazushige Adachi‡, Yukihiro Nishio‡, Jun Hayakawa‡, Seiji Mabuchi‡, Kazuhiro Takahashi‡, Keiichi Tsaka‡, Yasuhide Miyamoto‡, Naoyuki Taniguchi‡, and Yuji Murata‡

From the ‡Department of Obstetrics and Gynecology and ¶Department of Biochemistry, Osaka University Medical School, 2-2, Yamadaoka, Suita, Osaka 565-0871, Japan

Raloxifene is a tissue-selective estrogen receptor modulator. The effect of estrogen on cardiovascular disease is mainly dependent on direct actions on the vascular wall involving activation of endothelial nitric oxide synthase (eNOS) via Akt and extracellular signal-regulated protein kinase (ERK) cascades. Although raloxifene is also known to activate 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 and simian virus 40-transformed rat lung vascular endothelial cells (TRLECs), the raloxifene analog LY117018 caused acute phosphorylation of eNOS that was unaffected by actinomycin D and was blocked by the pure estrogen receptor antagonist ICI182,780. Activation of Akt by raloxifene reached a plateau at 15–30 min and declined thereafter, a similar time frame to that of Akt activation by 17β-estradiol. On the other hand, both activation and phosphorylation of ERK by raloxifene showed a biphasic pattern (peaks at 5 min and 1 h), whereas ERK activation and phosphorylation by 17β-estradiol reached a plateau at 5 min and declined thereafter. A MEK inhibitor, PD98059, had no effect on the raloxifene-induced Akt activity, suggesting an absence of cross-talk between the ERK and Akt cascades. Either exogenous expression of a dominant-negative Akt or pretreatment of TRLECs with PD98059 decreased the raloxifene-induced eNOS phosphorylation. Moreover, raloxifene stimulated the activation of Akt, ERK, and eNOS in Chinese hamster ovary cells expressing estrogen receptor α but not Chinese hamster ovary cells expressing estrogen receptor β. Our findings suggest that raloxifene-induced eNOS phosphorylation is mediated by estrogen receptor α via a nongenomic mechanism and is differentially mediated by Akt- and ERK-dependent cascades.

Heart disease is the most common cause of death of women in most industrialized nations, and observational studies have suggested a 50% reduction in heart disease risk in postmenopausal women taking estrogen (3). The mechanism of this cardiovascular protective effect has not yet been established, but favorable effects of hormone therapy on plasma lipids and vascular endothelial function have been proposed. However, estrogens can also increase the risk of breast cancer (4, 5) and uterine endometrial hyperplasia and cancer (4). Combining estrogen with progesterone has been shown to decrease the incidence of uterine endometrial hyperplasia and the risk of uterine endometrial cancer, but unfortunately it also lessens the beneficial effects of estrogen on lipid profiles, and it does not ameliorate the increased risk of breast cancer (3).

There is, therefore, a need for an ideal estrogen. The search for such an ideal compound has led to the development of a class of drugs termed selective estrogen receptor modulators (SERMs) (6). Raloxifene is a nonsteroidal benzo-thiophene that has been classified as a SERM based on the facts that it produces both estrogen-agonistic effects on bone (7) and lipid metabolism (6) and estrogen-antagonistic effects on uterine endometrium (7) and breast tissue (9, 10). Because of its tissue selectivity, raloxifene may cause fewer side effects than are typically observed with estrogen therapy. Raloxifene has been approved by the United States Food and Drug Administration for the prevention and treatment of osteoporosis. However, its ability to protect against cardiovascular disease has yet to be determined and awaits the results of the RUTH (raloxifene use for the heart) trial (11). Future experimental studies will be needed to address the cardioprotective effects of raloxifene.

Recent evidence suggests that the direct actions of estrogen on blood vessels contribute to the cardioprotective effects of estrogen (12, 13). 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 of the development of atherosclerosis. Several studies suggest that a key mediator of this vasodilator response may be the endothelium-derived relaxing factor nitric oxide (NO) (14). Estrogen activates endothelial nitric oxide synthase

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§ To whom correspondence should be addressed. Tel.: 81-6-6879-3354; Fax: 81-6-6879-3359; E-mail: masa@gyne.med.osaka-u.ac.jp.
(eNOS) without altering expression of the eNOS gene in vascular endothelium (14, 16–18). 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 is an important regulator of various cellular processes including glucose metabolism and cell survival (19, 20). Akt was identified as a downstream component of survival signaling through phosphatidylinositol (PI) 3-kinase (21–25). Activated Akt can phosphorylate substrates such as glycogen synthase kinase 3, 6-phosphofructo-2-kinase, and Bcl-2-associated death promoter. It was found that eNOS is also an Akt substrate and is activated by Akt-dependent phosphorylation, leading to release of NO in endothelial cells (26–29). More recently, we (30) and another group (31) showed that estrogen induces the Akt-dependent activation of eNOS in vascular endothelial cells (30, 31). In addition, evidence of a short term activation of eNOS, mediated by estrogen receptor-α functioning in a nongenomic manner via mitogen-activated protein kinase, has recently been published (17).

Recent findings have shown that raloxifene induces endothelium-dependent vasodilation (33) and directly activates NO release from endothelial cells (34). Raloxifene has the ability to induce eNOS phosphorylation without significantly altering expression of the eNOS gene in vascular endothelial (14, 16–18). More recently, the actions of estrogen can be mediated by the classical nuclear receptors estrogen receptor (ER) α and ERβ (36, 37) or through other putative membrane receptors. Therefore, we sought to determine whether the eNOS phosphorylation induced by the raloxifene analog LY117018 is mediated by Akt activation or determine whether the eNOS phosphorylation induced by the other putative membrane receptors. Therefore, we sought to determine whether the eNOS phosphorylation induced by the raloxifene analog LY117018 is mediated by Akt activation or determine whether the eNOS phosphorylation induced by the raloxifene analog LY117018 is mediated by Akt activation or whether eNOS phosphorylation induced by the other putative membrane receptors acting via a nongenomic mechanism. We previously demonstrated that the expression of eNOS in both human umbilical vein endothelial cells (HUVECs) and simian virus 40-transformed rat lung vascular endothelial cells (TRLECs) (38).

**EXPERIMENTAL PROCEDURES**

Materials—Raloxifene analog (LY117018) was a kind gift from Eli Lilly Research Laboratories (Indianapolis, Indiana). 17β-E2, actinomycin D, wortmannin, and nolvasen basic protein were purchased from Sigma. ICI 182,780 was obtained from TOCRIS (Baltimore, MD). ECL Western blotting detection reagents were obtained from Amersham Pharmacia Biotech. An Akt kinase assay kit including GSK-3 fusion protein. The kinase reaction was allowed to proceed at 30 °C for 30 min and stopped by the addition of Laemmli SDS sample buffer (47). Reaction products were resolved by 15% SDS–PAGE and transferred to nitrocellulose membranes. The membranes were blocked in 5% nonfat dry milk in TBS and incubated for 1 h at room temperature with a 1:1000 dilution of either rabbit polyclonal anti-eNOS antibody or mouse monoclonal anti-phospho ERK1/2 antibody. The membranes were washed twice with TBS and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The membranes were washed again and incubated with chemiluminescent reagents for 5 min at room temperature. The membranes were then exposed to X-ray film (Kodak X-OMAT R-250) and subjected to autoradiography to confirm that the detected bands were phosphorylated eNOS.

**Assay of eNOS Phosphorylation—**TRLECs or HUVECs were cultured in 60-mm dishes. 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 containing 20 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 5 mM sodium vanadate, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM sodium molybdate, and 2 mM dithiothreitol (30) containing 5 μg of partially purified eNOS and 40 μM [γ-32P]ATP (1 μCi). In some experiments, TRLECs were cultured in 100-mm dishes. Serum-deprived cells were incubated with 10−6 M raloxifene for 15 min, and lysates were prepared from the cells as described above and subjected to immunoprecipitation with anti-Akt or anti-ERK1 antibody. The immunoprecipitated products were washed once with lysis buffer and twice with kinase assay buffer and resuspended in 30 μl of kinase assay buffer containing 5 μg of partially purified eNOS and 40 μM [γ-32P]ATP (1 μCi). The kinase reaction was allowed to proceed at room temperature for 5 min and stopped by the addition of Laemmli SDS sample buffer (47). Reaction products were resolved by 8% SDS–PAGE and subjected to Western blotting (50) with an anti-phospho-GSK-3 antibody. The immunoprecipitated products were then washed twice with phosphate-buffered saline and lysed in ice-cold HNTG buffer (50 mM HEPES, pH 7.5, 10 mM KCl, 1 mM EDTA, 1 mM orthovanadate, 1 μg/ml leupeptin, and 1 μM phenylmethylsulfonyl fluoride) (30). The extracts were centrifuged to remove cellular debris, and the protein content of the supernatants was determined using the Bio-Rad protein assay reagent. Thirty μg of lysates were mixed with 30 μl of kinase assay buffer (25 mM Tris, pH 7.5, 1 μg/ml leupeptin, 5 mM sodium fluoride, 1 mM sodium orthovanadate, 1 μg/ml pepstatin, and 2 mM dithiothreitol) (30) containing 5 μg of partially purified eNOS and 40 μM [γ-32P]ATP (1 μCi). In some experiments, TRLECs were cultured in 100-mm dishes. Serum-deprived cells were incubated with 10−6 M raloxifene for 15 min, and lysates were prepared from the cells as described above and subjected to immunoprecipitation with anti-Akt or anti-ERK1 antibody. The immunoprecipitated products were washed once with lysis buffer and twice with kinase assay buffer and resuspended in 30 μl of kinase assay buffer containing 5 μg of partially purified eNOS and 40 μM [γ-32P]ATP (1 μCi). The kinase reaction was allowed to proceed at room temperature for 5 min and stopped by the addition of Laemmli SDS sample buffer (47). Reaction products were resolved by 8% SDS–PAGE and subjected to Western blotting (50) with an anti-phospho-GSK-3 antibody.

**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 containing 20 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 5 mM sodium vanadate, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM sodium molybdate, and 2 mM dithiothreitol (30) containing 5 μg of partially purified eNOS and 40 μM [γ-32P]ATP (1 μCi). The kinase reaction was allowed to proceed at room temperature for 30 min and stopped by the addition of Laemmli SDS sample buffer (47). Reaction products were resolved by 8% SDS–PAGE and subjected to Western blotting (50) with an anti-phospho-GSK-3 antibody.

**Preparation of Partially Purified eNOS—**Human eNOS was overexpressed in SF-21 cells, which had been infected with baculovirus carrying human eNOS cDNA (45). Human eNOS was partially purified by chromatography on 2.5% ADP-Sepharose gel, and its specificity was determined as described previously (46).
HUVECs (lower panel) were treated with $10^{-6}\text{M}$ raloxifene for 15 min and then were treated with $10^{-6}\text{M}$ raloxifene for 15 min. eNOS phosphorylation was measured using purified eNOS as a substrate as described under “Experimental Procedures.” Autoradiograms of $^{32}\text{P}$-labeled eNOS are shown. Experiments were repeated three times with essentially identical results. In B, TRLECs were pretreated with $10^{-6}\text{M}$ ICI182,780 for 15 min (lane 3) or $25\mu\text{g/ml}$ actinomycin D (Act-D) for 120 min (lane 4) and then were treated with $10^{-6}\text{M}$ raloxifene for 15 min. eNOS phosphorylation was measured using purified eNOS as a substrate as described under “Experimental Procedures.” Autoradiograms of $^{32}\text{P}$-labeled eNOS are shown in the lower panel. Relative densitometric units of the phosphorylated eNOS bands are shown in the upper panel, with the density of the control band set arbitrarily at 1.0. Values shown represent the mean ± S.E. from at least three separate experiments. Significant differences are indicated by asterisks. **, $p<0.01$ as compared with the control.

**RESULTS**

**Induction of eNOS Phosphorylation by Raloxifene**—To evaluate whether eNOS phosphorylation is induced (Fig. 1A) by raloxifene in TRLECs (upper panel) and HUVECs (lower panel), cultured cells were exposed to raloxifene for the indicated times. The increase in eNOS phosphorylation induced by $10^{-6}\text{M}$ raloxifene reached a plateau at 5–15 min in TRLECs or at 15–30 min in HUVECs and rapidly declined thereafter. To determine whether this response involves rapid ER activation, the effect of concomitant treatment with the pure ER antagonist ICI182,780 was determined. ICI182,780 significantly decreased the induction of eNOS phosphorylation by raloxifene in TRLECs (Fig. 1B, lane 3). Moreover, the effects of actinomycin D, an inhibitor of gene transcription, were tested to rule out the influence of genomic events mediated by nuclear ERs (Fig. 1B). Actinomycin D did not affect the induction of eNOS phosphorylation by raloxifene (Fig. 1B, lane 4).

**Activation of Akt by Raloxifene**—To determine whether Akt is activated by raloxifene in TRLECs, raloxifene was added to cultured cells for the indicated times (Fig. 2A) and at the indicated concentrations for 15 min (Fig. 2B). 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 raloxifene reached a plateau at 15–30 min and declined thereafter (Fig. 2A). Raloxifene induced the activation of Akt in a dose-dependent manner (Fig. 2B). Because Akt is an effector of survival signaling downstream of PI 3-kinase (21–25), we next examined whether stimulation with raloxifene increased the activity of Akt through a PI 3-kinase-dependent mechanism. TRLECs were stimulated with raloxifene in the presence or absence of wortmannin, a PI 3-kinase inhibitor, and the kinase activity of Akt was assayed. The induction of Akt activity by raloxifene was inhibited by wortmannin (Fig. 3, lane 5). These results indicate that raloxifene activates Akt activity through a PI 3-kinase-dependent mechanism.

To determine whether this process involves rapid ER activation, the effect of concomitant treatment with the pure ER antagonist ICI182,780 was determined (Fig. 3, lane 3). ICI182,780 clearly caused a decrease in raloxifene-induced Akt activation. Moreover, actinomycin D, an inhibitor of gene transcription, was used to rule out the influence of genomic events mediated by nuclear ERs. Actinomycin D did not affect the induction of Akt activity by raloxifene (Fig. 3, lane 4).
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**Activation of Akt by raloxifene in endothelial cells.** TRLECs were grown in 100-mm dishes. In A, cells were treated with 10⁻⁶ M raloxifene for the indicated times. In B, cells were treated with the indicated concentrations of raloxifene for 15 min. Lysates were subsequently subjected to immunoprecipitation (I.P.) with immobilized anti-Akt (A-Akt) antibody, and the kinase reaction was carried out in the presence of ATP and GSK-3α fusion protein as described under "Experimental Procedures." After the reactions were stopped with Laemml sample buffer, samples were resolved by 12% SDS-PAGE and then analyzed by Western blotting with an anti-phospho-GSK-3α antibody. Experiments were repeated three times with essentially identical results. C, control.

**Fig. 2.** Activation of Akt by raloxifene in endothelial cells. TRLECs were grown in 100-mm dishes. In A, cells were treated with 10⁻⁶ M raloxifene for the indicated times. In B, cells were treated with the indicated concentrations of raloxifene for 15 min. Lysates were subsequently subjected to immunoprecipitation (I.P.) with immobilized anti-Akt (A-Akt) antibody, and the kinase reaction was carried out in the presence of ATP and GSK-3α fusion protein as described under "Experimental Procedures." After the reactions were stopped with Laemml sample buffer, samples were resolved by 12% SDS-PAGE and then analyzed by Western blotting with an anti-phospho-GSK-3α antibody. Experiments were repeated three times with essentially identical results. C, control.

**Fig. 3.** Mechanism of augmentation of Akt activation by raloxifene. TRLECs were grown in 100-mm dishes. Cells were pretreated with 10⁻⁶ M ICI182,780 for 15 min (lane 3), 25 μg/ml actinomycin D (Act-D) for 120 min (lane 4), or 20 μM wortmannin for 15 min (lane 5) and then treated with 10⁻⁶ M raloxifene for 15 min. Akt activity was measured as described in the legend for Fig. 2. Experiments were repeated three times with essentially identical results. C, control. I.P., immunoprecipitates. A-, anti.-

**Akt-dependent eNOS Phosphorylation**—To determine whether raloxifene-induced Akt activation is involved in the phosphorylation of eNOS, 10⁻⁶ M raloxifene 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 (30, 46) as a substrate (Fig. 4A). Treatment of cells with raloxifene increased the phosphorylation of eNOS in anti-Akt immunoprecipitates (Fig. 4A, lanes 1–3). ICI182,780 caused a significant decrease in the raloxifene-induced phosphorylation of eNOS in anti-Akt immunoprecipitates (Fig. 4A, lane 4). Moreover, we evaluated the effect of exogenous expression of various forms of Akt on the in vitro phosphorylation of purified eNOS. TRLECs transfected with wild-type or mutant forms of HA-tagged Akt were exposed to 10⁻⁶ M raloxifene. First, we determined the effects of the expression of an Akt derivative rendered kinase-inactive by a point mutation within the Akt catalytic domain (HA-AktK179M) and an Akt derivative rendered constitutively active via targeting to the plasma membrane with a myristoyl tag (HA-mΔ4–129Akt) (40, 53) on Akt activity (Fig. 4B, i). Extracts from these cells were immunoprecipitated with anti-HA antibody and assayed for Akt activity. The expression of HA-AktK179M inhibited the raloxifene-induced Akt activity (Fig. 4B, I, lane 6), and the expression of HA-mΔ4–129Akt stimulated the basal Akt activity (Fig. 4B, i, lane 3). Next, extracts from these cells were immunoprecipitated with anti-HA antibody and assayed in an immunocomplex kinase assay for their ability to phosphorylate purified eNOS. Treatment of the cells with raloxifene increased the phosphorylation of eNOS in anti-HA immunoprecipitates prepared from TRLECs transfected with wild-type Akt (Fig. 4B, ii, lane 2) but not in anti-HA immunoprecipitates prepared from TRLECs transfected with the kinase-inactive Akt (Fig. 4B, ii, lane 6). In addition, anti-HA immunoprecipitates from TRLECs transfected with constitutively active Akt were found to contain elevated basal eNOS phosphorylation in immunocomplex kinase assays (Fig. 4B, ii, lane 3). These results suggest that the raloxifene-induced Akt activation causes increased phosphorylation of eNOS.

**Activity of ERK by Raloxifene**—Recently, it was reported that estrogen activates eNOS in a nongenomic manner via ERK activation in endothelial cells (17). We therefore investigated whether raloxifene induces the activation and/or the phosphorylation of ERK. Cells were treated with 10⁻⁷ M 17β-E2 or 10⁻⁶ M raloxifene for the indicated times. Cell lysates were immunoprecipitated with anti-ERK antibody and examined for either ERK activity by assaying the incorporation of 32P into myelin basic protein (MBP, Fig. 5B, i) or for ERK phosphorylation by Western blotting with anti-phospho-ERK antibody (Fig. 5B, ii). We confirmed that the total amount of ERK in each lane was the same (Fig. 5, iii). Both activation and phosphorylation of ERK by 17β-E2 reached a plateau at 5 min and declined thereafter (Fig. 5A). On the other hand, raloxifene-dependent increases in both ERK activity and ERK phosphorylation displayed a biphasic time course; the activity reached a maximum at 5 min, rapidly declined and increased again after 1 h of raloxifene stimulation, and declined thereafter (Fig. 5B). IC1182,780 clearly decreased the activation and the phosphorylation of ERK by 17β-E2 (Fig. 5A, lane 8) and raloxifene (Fig. 5B, lane 8).

**ERK-dependent eNOS Phosphorylation**—To determine whether raloxifene-induced ERK activation is involved in the phosphorylation of eNOS, 10⁻⁶ M raloxifene was added to cultured cells for 5 min or 1 h. Cell lysates were subjected to immunoprecipitation with anti-ERK1 antibody and then assayed in an immunocomplex kinase assay using purified eNOS (30, 46) as a substrate (Fig. 6A). Treatment of cells with raloxifene for 5 min increased the phosphorylation of eNOS in anti-ERK immunoprecipitates (Fig. 6A, lane 3). Moreover, we determined the effects of the expression of an Akt derivative rendered kinase-inactive by a point mutation within the Akt catalytic domain (HA-AktK179M) and an Akt derivative rendered constitutively active via targeting to the plasma membrane with a myristoyl tag (HA-mΔ4–129Akt) (40, 53) on Akt activity (Fig. 6B, i). Extracts from these cells were immunoprecipitated with anti-HA antibody and assayed for Akt activity. The expression of HA-AktK179M inhibited the raloxifene-induced Akt activity (Fig. 6B, I, lane 6), and the expression of HA-mΔ4–129Akt stimulated the basal Akt activity (Fig. 6B, i, lane 3). Next, extracts from these cells were immunoprecipitated with anti-HA antibody and assayed in an immunocomplex kinase assay for their ability to phosphorylate purified eNOS. Treatment of the cells with raloxifene increased the phosphorylation of eNOS in anti-HA immunoprecipitates prepared from TRLECs transfected with wild-type Akt (Fig. 6B, ii, lane 2) but not in anti-HA immunoprecipitates prepared from TRLECs transfected with the kinase-inactive Akt (Fig. 6B, ii, lane 6). In addition, anti-HA immunoprecipitates from TRLECs transfected with constitutively active Akt were found to contain elevated basal eNOS phosphorylation in immunocomplex kinase assays (Fig. 6B, ii, lane 3). These results suggest that the raloxifene-induced Akt activation causes increased phosphorylation of eNOS.
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**Fig. 4. Akt-dependent eNOS phosphorylation.** In A, TRLECs were pretreated or not pretreated with 10⁻⁸ m ICI182,780 for 15 min and then treated with 10⁻⁷ m raloxifene for the indicated times. Cell lysates were subjected to immunoprecipitation (I.P.) with anti-Akt antibody and then assayed in an immunocomplex kinase assay using purified eNOS as a substrate. Autoradiograms of ³²P-labeled eNOS immunoprecipitated with anti-Akt antibody are shown in the lower panel. Relative densitometric units of phosphorylated eNOS bands are shown in the upper panel, with the density of the control band set arbitrarily at 1.0. Values shown represent the mean ± S.E. from at least three separate experiments. Significant differences are indicated by asterisks. **, p < 0.01 as compared with the control. C, control. In B, the effect of expressed Akt on the activation of Akt (i) or the phosphorylation of purified eNOS (ii) induced by raloxifene was examined. Immunocomplex kinase assays were performed using anti-HA immunoprecipitates from cells expressing HA-tagged Akt constructs encoding HA-Akt (wild-type Akt), kinase-inactive HA-Akt/K179M (inactive Akt), or constitutively active HA-mAktΔ129Akt (active Akt) expressed in TRLECs using GSK-3β as a substrate (i) or purified eNOS as a substrate (ii). Relative densitometric units of phosphorylated eNOS bands are shown in the upper panel of ii, with the density of the control band set arbitrarily at 1.0. Values shown represent the mean ± S.E. from at least three separate experiments. Significant differences are indicated by asterisks. **, p < 0.01 as compared with the control. A-, anti-; I.P., immunoprecipitates.

**Fig. 5. Activation and phosphorylation of ERK by raloxifene in endothelial cells.** TRLECs were grown in 100-mm dishes. Cells were treated with 10⁻¹⁰ m 17β-E2 (A) or 10⁻⁷ m raloxifene (B) for the indicated times. Lysates were subsequently immunoprecipitated with anti-ERK1 antiserum. In i, the immunoprecipitates were incubated with [γ-³²P]ATP in the presence of myelin basic protein (MBP) as described under “Experimental Procedures.” After the reactions were stopped by the addition of Laemmli sample buffer, samples were subjected to SDS-PAGE and autoradiographed. The immunoprecipitates were subjected to Western blotting with anti-phospho-ERK antibody (ii) or anti-ERK1 antibody (iii) as described under “Experimental Procedures.” Experiments were repeated three times with essentially identical results. A-, anti-; I.P., immunoprecipitates.

**Effect of ERα or ERβ Expression on the Raloxifene-induced Activation of Akt and ERK and the Phosphorylation of eNOS.** The potential roles of ERα and ERβ in raloxifene-induced Akt and ERK activation were evaluated. For this, CHO cells, which do not express ERα or ERβ (56), were transfected with expression vectors for these receptors. Transfection of ERβ expression vector had no effect on raloxifene-induced Akt (i) or ERK (ii) activation compared with transfection of control vector (Fig. 7A). On the other hand, transfection of ERα expression vector caused increases in raloxifene-induced Akt (i) and ERK (ii) activation compared with transfection of control vector (Fig. 7A). Moreover, the potential roles of ERα and ERβ in raloxifene-induced eNOS phosphorylation were evaluated. In CHO

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cells transfected with control vector or ERβ expression vector, raloxifene had no effect on eNOS phosphorylation (Fig. 7B). However, in cells transfected with ERα expression vector, there was a clear increase in eNOS phosphorylation upon brief stimulation with raloxifene (Fig. 7B). These results indicate that raloxifene induces Akt and ERK activity and eNOS phosphorylation through ERα.

**DISCUSSION**

This study showed that Akt and ERK are differentially activated by raloxifene and that both the Akt and ERK activations contribute to the enhanced phosphorylation of eNOS after brief treatment with raloxifene; expression of inactive Akt by TRLECs or treatment with PD98059, a MEK inhibitor, attenuated the raloxifene-induced eNOS phosphorylation. Although the kinetics of raloxifene-induced Akt activation are similar to those of estrogen-induced Akt activation (30), the kinetics of raloxifene-induced ERK activation and phosphorylation are different from those of estrogen-induced ERK activation and phosphorylation (Fig. 5). A pure ER antagonist, ICI182,780, completely inhibited the raloxifene-induced Akt and ERK activation and eNOS phosphorylation. Moreover, in CHO cells transfected with an ERα expression vector, there were clear increases in Akt and ERK activities and eNOS phosphorylation upon brief stimulation with raloxifene. The present report is the first to show that raloxifene differentially activates PI-3 kinase-Akt and MEK-ERK cascades, associated with an increase in phosphorylation of eNOS through ERα.

eNOS is an important modulator of angiogenesis and vascular tone (57) and plays an important role in the direct effects of estrogen on blood vessels (58). eNOS activity is stimulated in endothelial cells in a PI-3 kinase-dependent fashion by treatment with insulin-like growth factor-1, vascular endothelial growth factor, or estrogen (59, 60). The protein kinase Akt is an important downstream target of PI 3-kinase (61). Akt phosphorylates substrates within a defined motif that is present in the sequence surrounding Ser-1177 in eNOS (62). Thus, the Akt-

![Fig. 6. ERK-dependent eNOS phosphorylation.](http://www.jbc.org/)

![Fig. 7. Effect of ERα or ERβ expression on raloxifene-induced Akt and ERK activation and eNOS phosphorylation.](http://www.jbc.org/)

![A](http://www.jbc.org/)

![B](http://www.jbc.org/)
PI-3 kinase cascade is involved in the mechanism of eNOS activation. The role of mitogen-activated protein kinases in eNOS regulation has also been evaluated. It was reported that bradykinin treatment of bovine aortic endothelial cells led to the activation of ERK, associated with an increase in the phosphorylation of eNOS (63). Additionally, ERK activation is known to be involved in estrogen induction of eNOS activity (17), specifically in the initial increase of estrogen-induced eNOS activity (31). However, the effect of SERMs on ERK/mitogen-activated protein kinase in endothelial cells remains unclear. The signal transduction mechanisms of tamoxifen, which is one of the SERMs, have been evaluated in other cells. In HeLa cells, tamoxifen activates both c-Jun NH2-terminal kinase1 and ERK2, which may play important roles in the regulation of gene expression by tamoxifen (64). Moreover, in breast cancer cells that do not express estrogen receptor, tamoxifen activates c-Jun NH2-terminal kinase and caspase and subsequently induces apoptosis (65). In this report, we demonstrated that raloxifene induced ERK activation and phosphorylation with different kinetics from estrogen and that these effects were associated with an increase in the phosphorylation of eNOS.

SERMs arestructurally diverse non-steroidal compounds that bind to estrogen receptors and produce estrogen agonist effects in some tissues and estrogen antagonist effects in others. Numerous studies have examined the molecular basis of SERM selectivity. SERM selectivity reflects the diversity of estrogen receptor forms and coregulators, cell type differences in the expression of these receptors and coregulators, and the diversity of ER target genes (66). There are two estrogen receptors, ERα and ERβ, both of which are members of the superfamily of steroid hormone receptors (36, 67). Genetic disruption of ERα in mice leads to lower levels of vascular nitric oxide (68). In addition, ERs can directly activate endothelial nitric-oxide synthase (17, 18, 30, 31). In addition to forming homodimers, ERs and ERβ can form heterodimers with each other (69), adding a further degree of complexity to the regulation of gene expression by estrogen in cells expressing both receptors. The expression of ERβ, but not ERα, is elevated after vascular injury in male rats (70). ERβ has properties that distinguish it from ERα in that transcription from an apolipoprotein 1 site is regulated by the SERMs tamoxifen and raloxifene in opposite directions via ERα and ERβ (71). We found that there were clear increases in Akt activity, ERK activity, and eNOS phosphorylation upon brief treatment with raloxifene in CHO cells transfected with ERα expression vector (Fig. 7). On the other hand, no stimulatory effect of raloxifene on Akt activity, ERK activity, or eNOS phosphorylation was detected in cells transfected with control vector or ERα expression vector (Fig. 7). These findings suggest that ERα is capable of mediating the acute response and rapid vasodilatation caused by raloxifene as it does in the case of 17β-E2 (30, 31). Thus, the presence of ERα in target cells may contribute to the differential regulation by estrogen and raloxifene of cardioprotective effects.

Is the cardioprotective effect of raloxifene similar to that of estrogen? The vascular accumulation of oxidized low-density lipoprotein cholesterol and consequent effects on vasomotility are recognized as pivotal events in the initiation and progression of atherosclerosis (72, 73). Raloxifene inhibits the in vitro oxidation of human low-density lipoprotein cholesterol more potently than estrogen (41). It was also reported that raloxifene took longer to induce vascular relaxation than estrogen took in comparable studies (33). The relaxant effect of raloxifene was not reversed over periods of up to 5 h in contrast to estrogen, with which initial precontraction levels were achieved after 30 min. Thus, these data suggest that raloxifene may provide more potent protection against cardiovascular disease than estrogen.

Although eNOS phosphorylation was transiently induced by treatment of cells with raloxifene (Fig. 1), the raloxifene-dependent increases in ERK activity and phosphorylation displayed a biphasic time course. ERK immunoprecipitates from cells treated with raloxifene for 1 h did not catalyze the phosphorylation of eNOS (Fig. 6). The consequences of the delayed increase of raloxifene-induced ERK activation are under investigation. It was reported that 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors caused preservation of ischemic vasculature by preventing apoptosis in endothelial cells (32). Because regulation of BAD phosphorylation at serine 112 by the Ras-mitogen-activated cascade has been reported (53), there is a possibility that the delayed increase of raloxifene-induced ERK activation might be involved in preventing apoptosis in endothelial cells. Moreover, it was reported that lysophosphatidylcholine enhances eNOS promoter activity via an ERK1/2-dependent pathway (15). Although raloxifene was reported to have no effect on the gene expression of eNOS (34), there is still a possibility that the delayed increase of raloxifene-induced ERK activation might be involved in regulating eNOS promoter activity. Thus, the mechanism of the atheroprotective effect of raloxifene might be more complicated than that of the atheroprotective effect of estrogen, resulting in a stronger atheroprotective effect of raloxifene than estrogen, as reported previously (33).

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