Molecular Basis of Cell Membrane Estrogen Receptor Interaction With Phosphatidylinositol 3-Kinase in Endothelial Cells

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Objective—Nontranscriptional signaling mechanisms mediate some of the biological effects of estrogen, such as the rapid actions on the blood vessels. By interacting with phosphatidylinositol 3-kinase (PI3K), estrogen receptor (ER) α leads to activation of protein kinase Akt and to subsequent increase in endothelial nitric oxide synthase activity. Because PI3K is mainly a cytoplasmic complex, we studied the cellular site of interaction between this enzyme and ERα, and we dissected the molecular mechanisms that mediate this interaction.

Methods and Results—By using cultured human saphenous vein endothelial cells, we found that cell membrane–bound ERα colocalizes with PI3K and may be responsible for PI3K activation. Furthermore, we characterized the subsequent steps in the activation of the PI3K/Akt signaling cascade, comparing the molecular events that follow insulin or estradiol activation of PI3K.

Conclusions—We provide novel evidence for an important role of nonnuclear estrogen receptor in rapid, nontranscriptional responses of human endothelial cells to estrogen. (Arterioscler Thromb Vasc Biol. 2003;23:●●●●●●.)

Key Words: estrogen ■ estrogen receptor ■ nontranscriptional signaling ■ phosphatidylinositol 3-kinase ■ endothelium

Estrogen signaling has traditionally been identified with the transcriptional control of target genes via the binding of nuclear estrogen receptors to genomic consensus sequences. Nonetheless, in the past few years, several biological actions of estrogen have been identified that are too rapid to be compatible with transcriptional mechanisms.

Estrogen has cardiovascular protective effects that largely depend on nontranscriptional regulation of the vessel wall. The most prominent nongenomic action of estrogen at this level is the induction of rapid vasorelaxation, which partially depends on the modulation of cell membrane ion channels in endothelial and smooth muscle cells. In vascular smooth muscle cells (VSMCs), estradiol treatment inhibits voltage-dependent L-type Ca2+ channels. 17β-estradiol also controls potassium efflux in VSMCs by opening Ca2+- and voltage-activated K+ channels via cGMP-dependent phosphorylation. However, a major role is played by acute activation of NO synthesis in endothelial cells, which mediates estrogen effects also in humans.

Rapid induction of NO synthesis by estrogen largely depends on activation of the endothelial isoform of NO synthase (eNOS). Estrogen receptor (ER) α is involved in this phenomenon, which is in part attributable to mitogen-activated protein (MAP) kinases or tyrosine kinase activation.

In addition, we recently described the interaction of ERα with phosphatidylinositol 3-kinase (PI3K), showing that this mechanism accounts for the major part of eNOS activation in human endothelial cells. Moreover, in a mice model, estrogen decreases vascular leukocyte accumulation after ischemia/reperfusion injury in an eNOS-, PI3K-, and ER-dependent manner, and nongenomic recruitment of PI3K has marked anti-ischemic effects in a myocardial infarction model, confirming the pathophysiological importance of this nongenomic pathway.

PI3K is a lipid kinase mediating the cellular effects of cell membrane–bound receptor-dependent molecules. PI3K is predominantly a heterodimer formed by an 85-kDa (p85α) adapter/regulatory subunit and by a 110-kDa (p110) catalytic subunit. By phosphorylating the D-3 position of the phosphatidylinositol ring, PI3K synthesizes phosphatidylinositol 3-phosphates (PtdIns-3-P, PtdIns-3,4-P2, PtdIns-3,4,5-P3), which regulate the activity of kinases containing pleckstrin homology domains such as phosphatidylinositol-dependent kinases and protein kinase Akt. The serine/threonine kinase Akt represents the principal downstream effector of PI3K, triggering several of its cellular effects, including activation of eNOS.

The site of interaction between PI3K and ERα is unclear. Because PI3K is mainly cytoplasmic, it may be possible that cytoplasmic or cell membrane–bound ERs are responsible for the recruitment of PI3K. Indeed, extranuclear ERs have been described long since, and, very recently, membrane-bound
ERs have been identified in endothelial cells and implicated in the regulation of NO production. Moreover, there is lack of information on the specific molecular mechanisms that accompany PI3K activation on interaction with ERα and on the signaling apparatus that is recruited by estrogen.

The aim of this study was therefore to characterize the subcellular site of interaction of ERα and PI3K as well as the specific mechanisms through which ER couples to and transactivates PI3K.

Methods

Cell Cultures

Human saphenous and bovine aortic endothelial cells (BAECs) were harvested with type Ia collagenase. MCF-7 cells were from ATCC, and p85α+/− mice fibroblasts were a gift of Dr L. Cantley. All cells were cultured and stimulated under serum-starved conditions consisting of phenol red-free medium 199 or DMEM (Gibco BRL, Life Technologies) with 0.4% charcoal-stripped FCS.

Nitrite and eNOS Activity Assays

NO accumulation was determined by a modified nitrate assay using 2,3-diaminonaphthalene, as described. Endothelial cells were harvested in PBS containing 1 mmol/L EDTA, and cell lysates were assayed for eNOS activity, as described.

Immunoprecipitations and PI3K Assays

Endothelial cell protein extracts were IP’d with Abs versus progesterone receptor (Santa Cruz, clone C-20), IRS-1 (Santa Cruz, clone E-12) or IRS-2 (Santa Cruz, clone M-19), ERα (Neomarkers, clone TE111), eNOS (Transduction Laboratories, clone 3) or p85α (Pharmingen, clone U15), as described. The immunoprecipitates were either used for immunoblotting or for PI3K assays, as described. The labeled phospholipids were extracted with chloroform/methanol, and the organic phase, containing the PI3K products, was separated by borate TLC according to Walsh.

Immunoblotting

Endothelial cell lysates were separated by SDS-PAGE, and immunoblotting were performed with standard technique. The Abs used were progesterone receptor (Santa Cruz, clone C-20), p85α (Pharmingen, clone U15), P-Tyr (Santa Cruz, clone PY99), Goα (Santa Cruz, clone E-17), Sp1 (Santa Cruz, clone PEP 2), ERα (Neomarkers, clone TE111), wild-type Akt (catalogue No. 06-558) and Thr245/246-P-Akt (catalogue No. 06-678) or Ser423/424-P-Akt (catalogue No. 06-801) (all from Upstate Biotechnology), eNOS (Transduction Laboratories, clone 3), inducible NO synthase (iNOS) (Transduction Laboratories, clone 6), wild-type ERK 1/2 (catalogue No. 442704), or Tyr202-P-ERK 1/2 (catalogue No. 442705) (Calbiochem).

Transfection Assays

The Akt constructs have been described previously. BAECs and murine wild-type fibroblasts were transfected using the Lipofectamine reagent (Gibco BRL). To control for transfection efficiency, pcMV.β-Gal plasmid containing the β-galactosidase gene was cotransfected in all experiments. β-galactosidase staining indicated that transfection efficiency was 30% to 35%. Cells (60% to 70% confluent) were assayed for eNOS and β-galactosidase activities as described.

Statistical Analysis

All values are expressed as mean±SD. Statistical differences between mean values were determined by ANOVA, followed by the Fisher’s protected least-significance difference test for comparison of mean values. Two-group comparisons were performed by the unpaired Student’s t test.

A detailed Methods section is available online at http://atvb.ahajournals.org.

Results

In human endothelial cells, physiological concentrations of 17β-estradiol (E2) acutely increase NO release via an ER and PI3K-dependent mechanism. Because many effects of estrogen depend on transcriptional regulation, we investigated the effect of RNA synthase inhibitors, actinomycin D and 5,6-dichlorobenzimidazole riboside (DRB), and protein synthesis inhibitor, cycloheximide, on estrogen-induced eNOS activity. Actinomycin D, DRB, or cycloheximide have no effect on basal or E2-stimulated eNOS activity (Figure 1B).
The increase in ERα-associated PI3K activity is linked to increased association between ERα and the adapter/regulatory subunit p85α (Figure IIA). This interaction is specific for ERα, because no p85α coimmunoprecipitation can be detected with progesterone receptor (Figure IIB). Estrogen induces ERα-p85α interaction similarly to insulin-triggered association between the phosphotyrosine adapter insulin receptor substrate-1 (IRS-1) and p85α (Figure 2A). However, ERα-p85α interaction is not mediated by IRS-1 or IRS-2, because estrogen treatment is not associated with IRS-1/2 tyrosine phosphorylation (Figure 2B).

Because ERs are prevalently localized in the nucleus, whereas PI3K is mainly cytoplasmic, the site of interaction is unclear, and the adapter/p85α-bound PI3K. However, only a minority of PI3K associates with ERα. In fact, we did not detect changes in PI3K activity in the total p85α immunoprecipitates, as opposed to ERα coimmunoprecipitates (Figure IIC). In agreement with this data on eNOS, PI3K activation by E2 is independent of gene transcription or protein synthesis, as shown with DRB and cycloheximide (Figure IID). Confirming the immunoprecipitation studies, E2 does not recruit PI3K via IRS-1/2, because no activation of IRS-1/2-associated PI3K can be seen on estrogen challenge (Figure 2C). Furthermore, insulin is unable to trigger the activation of ERα-associated PI3K, nor to increase estrogen-induced PI3K activation (Figure 2D), suggesting that the 2 hormones recruit PI3K through distinct mechanisms.

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Because cell-membrane ERs have been described in endothelial cells, and they have been involved in eNOS regulation, we studied whether the membrane-impermeable E2-BSA complex was able to activate eNOS. Compared with E2, E2-BSA is still able to activate eNOS, but the kinetics are different (Figure 3D). Indeed, the early eNOS activation, which is sensitive to MAP kinase inhibitors, is comparable, but the later, wortmannin-sensitive increase is reduced (Figure 3D). From these experiments, there is evidence that cell membrane-bound estrogen receptors are important for binding and activation of PI3K.

One of the prominent downstream targets of PI3K is phosphorylation and activation of protein kinase Akt by PDK-1 and PKA. 2,14,15 Estrogen increases Akt kinase activity, but the mechanism is unclear. Akt is activated by 2 independent phosphorylations on serine47315 and threonine308.14 Under basal conditions, there is little threonine or serine phosphorylation of Akt (Figure 4A). Compared with E2 treatment, ERα staining in untreated cells is mostly nuclear, but after E2 treatment, ERα staining in the cytoplasmic/cell membrane compartment increases (Figures 3A and 3B) time-consistently with eNOS and PI3K activation.

Accumulation of ERα in the cytoplasm/cell membrane is prevented by tamoxifen (Figures 3A and 3B) and seems to be a feature of endothelial cells, because it is not detectable in MCF-7 cells in similar conditions (Figure 3B). PI3K staining is not affected by E2, but E2 favors the colocalization of ERα and p85α in the cytoplasmic/cell membrane compartment (Figure 3A), although a nuclear interaction cannot be completely excluded by these experiments. To confirm these observation, we obtained purified nuclear and cytoplasmic/cell membrane fractions from endothelial cells and studied the distribution of ERα and p85α.

The purity of the fractions was confirmed by immunoblotting for the detection of associated p85α and p85α (respectively), and then immunoblotted for the detection of associated p85α. B, HSVECs were treated for different times with E2 (10 nmol/L), with insulin (100 nmol/L), or with the anti-IRS-1 and anti-IRS-2 Abs, and then immunoblotted for the detection of IRS-1/2 tyrosine phosphorylation with a specific Ab. C, Effect of E2 (10 nmol/L, 30 minutes) or insulin (100 nmol/L, 30 minutes) on IRS-1/2-associated PI3K activity in HSVECs. D, Effect of insulin (100 nmol/L, 30 minutes) on basal or on estrogen-activated ERα-associated PI3K in HSVECs.

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4A). E₂ causes Akt threonine and serine phosphorylation in a time-delayed manner similar to E₂-stimulated PI3K and eNOS activation. Phosphorylation of Akt by E₂ is inhibited by wortmannin and ICI 182,780 (Figures 4A). These results demonstrate that activation of PI3K by ERα and H9251 is associated with phosphorylation of Akt, which represents the mechanism for Akt activation by estrogen. To understand if Akt phosphorylation may happen in the microenvironment of a complex involving ERα and PI3K, we performed immunoprecipitations for these 2 latter molecules and examined whether Akt coprecipitates with PI3K.

Figure 3. Cell membrane/cytoplasmic ERα is responsible for interaction with p85α. A and B, HSVECs (A and B) and MCF-7 (B) cells were stimulated with E₂ (10 nmol/L) with and without tamoxifen (TM, 1 μmol/L), and immunofluorescent staining using antibodies to ERα (FITC, green) or p85α (rhodamine, red), alone or in combination, was performed. Note that between 10 and 20 minutes after E₂ stimulation, there is increased ERα staining in the cytoplasm/cell membrane of endothelial cells, where it colocalizes with p85α (arrows), whereas this does not happen in MCF-7 cells (B). C, Purified cytoplasmic/cell membrane or nuclear protein extracts from HSVECs treated with E₂ (10 nmol/L) or with vehicle (ethanol) were immunoblotted for the membrane-associated G protein Gαq, for the nuclear-associated Sp1, for ERα or for p85α. D, Effect of cell membrane impermeable BSA-conjugated E₂ (E₂-BSA) (10 nmol/L) versus E₂ (10 nmol/L) on eNOS activation in HSVECs in the presence or absence of wortmannin (WM, 30 nmol/L) or ICI 182,780 (ICI, 10 μmol/L). *P<0.05 compared with time-corresponding E₂-BSA only–treated cells.

Figure 4. Activation of Akt through Ser/Thr phosphorylation mediates estrogen–induced eNOS activation. A, Effect of 17β-estradiol (E₂, 10 nmol/L), 17β-estradiol (αE₂, 10 nmol/L), or insulin (Ins, 100 nmol/L) on the serine-threonine phosphorylation of Akt in the presence or absence of ICI 182,780 (ICI, 10 μmol/L) or wortmannin (WM, 30 nmol/L) on eNOS activation in HSVECs. The experiment was performed 3 times with similar results. B, Effect of transfection of empty vector, wild-type Akt, or constitutively active or negative dominant Akt constructs in BAECs (shown in the middle box) on ERα, p85α, eNOS, or iNOS protein expression. Two separate experiments yielded similar results.
them. Our results show that this is not the case (Figure IIIA), suggesting that Akt phosphorylation and eNOS activation take place as separate processes respect to ERα/PI3K interaction. This hypothesis is additionally supported by the evidence that ERα and p85α do not associate with eNOS in basal conditions nor after E2 treatment (Figure IIIB).

Akt mediates eNOS activation by estrogen, as confirmed by the transient transfection of BAECs and murine fibroblasts with Akt mutant constructs. BAECs express both ERα and eNOS, and the p85α+/− fibroblasts were cotransfected with ERα and eNOS cDNAs. The transfection of the wild-type form of Akt or of the two myristylated, constitutively active Akt mutants (myr-Akt and ΔPH-myr-Akt [deletion of the pleckstrin homology domain]) markedly increased eNOS activity (Figure IIIC). Transfection of a kinase-inactive dominant-negative Akt mutant with a point mutation in the ATP binding domain, Akt (K179M), does not affect basal eNOS activity but decreases E2-stimulated eNOS activity by 50% (Figure IIIC), compatible with transfection efficiency. Because transfection experiments require longer periods of incubation in the presence of the constructs, we checked whether transfection itself may be inducing eNOS or iNOS expression or alter ERα or p85α levels. BAECs overexpressing the different Akt constructs have unchanged amounts of ERα, p85α, and eNOS, and no expression is present for iNOS in any condition (Figure 4B). These experiments confirm that acute regulation of eNOS by Ser/Thr-phosphorylated Akt accounts for the rapid production of NO after estrogen treatment in endothelial cells.

**Discussion**

The role of cell membrane–bound estrogen receptors has been discussed for several years, since the first identification of membrane binding sites for 17β-estradiol. Since then, estrogen receptors localized on the cell membrane have been proposed to be involved in the transduction of the nongenomic effects of estrogen, that is with that variety of actions that estrogen exerts in different tissues, which are too rapid to be compatible with gene transcription and protein synthesis. Nonetheless, there is still uncertainty about the real nature of these receptors, as well as about the signaling mechanisms that they activate on binding with estrogen.

Several intracellular signaling cascades have been associated with rapid estrogen-dependent effects: the adenylate cyclase pathway, the phospholipase C pathway, the G-protein-coupled receptor-activated pathways, as well as the MAP kinase pathway.

At the vascular level, cell-surface ERs have been proposed to mediate estrogen-dependent rapid activation of NO synthesis, and this seems to be partially dependent on MAP kinase activation. We have shown that rapid eNOS regulation by estrogen in endothelial cells is played through modulation of the PI3K/Akt pathway via a direct interaction of ERα with the regulatory subunit of PI3K, p85α.

Our present data add to these findings, defining a role for cell-membrane estrogen receptors as the potential subpopulation of ERs that interacts with PI3K. These findings significantly broaden the role of cell membrane ERs, potentially involving these receptors with a variety of intracellular activities triggered by this lipid kinase.

The debate is open on the origin of cell membrane ERs. Recently, transfection studies have shown that nuclear and cell membrane ERs derive from the same transcripts, but still no data are available on possible conformational differences between these subpopulations. We have shown that ERα interacts with p85α on a conformational change dependent on estradiol binding and that this phenomenon does not require adapter molecules. Our present data show that BSA-conjugated E2 activates eNOS through PI3K/Akt. However, we find differences in the profile of eNOS activation when comparing the effects of E2-BSA versus natural E2. A possible explanation may be that the steric hindrance imposed by the BSA may partially prevent the association between the engaged ERα and p85α, supporting the concept that, as for ER-dependent nuclear effects, a correct conformational change on ligand binding is necessary for the nongenomic signaling of cell membrane–bound ERs. Alternatively, it may be that part of the ERα/PI3K interaction takes place in the cytoplasm, and therefore E2-BSA may be less potent than E2 because of lack of recruitment of non–membrane-bound cytoplasmic ERα.

An intriguing finding is the apparent increase in the ERα amount in the cytoplasm after estradiol exposure in endothelial but not MCF-7 cells. Although the mechanisms that regulate steroid receptors cytoplasmic/nuclear shuttling are not completely clear, there are hints that interaction with specific coactivators or the phosphorylation status of RNA polymerase II C-terminal domain may induce cyclic assembly and disassembly of ER transcription complexes in the nucleus, therefore inducing ER cycling on and off the nucleus, which may possibly serve to create a frequent sampling of the extracellular hormonal milieu. If this is the case, cell-specific coactivators may induce preferential ER cytoplasmic or nuclear localization and may provide the basis for the different relevance of nongenomic versus genomic signaling of ERα in distinct tissues.

We provide evidence that ERα recruits PI3K independently by adapter molecules mediating insulin signaling, such as IRS-1/2 or by tyrosine kinases pathways, therefore additionally characterizing the molecular events that link ERα to PI3K. We also show that interaction with PI3K is specific for ERα and does not extend to progesterone receptor. ERβ does not interact with PI3K, as well, and this reinforces the possibility that the two isoforms may have partially distinct roles in some tissues, such as the vascular wall. This has been previously shown using vascular injury models, where estrogen protective effects are entirely mediated by ERα, although ERβ (and not ERα) expression increases steeply after the injury, suggesting distinct roles for the 2 receptors. The differential interaction with PI3K may therefore represent the first example of the different role of ERα and ERβ on nongenomic signaling pathways.

When looking at the amount of PI3K activated by ERs, it turns out that the recruitment of a small fraction of total PI3K is sufficient to give rise to the visible regulatory effects that ensue on estrogen treatment of endothelial cells (ie, eNOS activation and NO release). This is strongly suggested by the fact that modulation of ERα-mediated PI3K activity is not discernible in p85α-immunoprecipitates, because of the high basal activity contributed by total cellular PI3K. Thus, because most of PI3K is potentially active and resides in the cytoplasm under basal conditions, ERα-associated PI3K must have some specificity to
activate the signal for eNOS activation. A possible explanation for this may be represented by the localization to the cell membrane of the ER subpopulation, which may favor signal transduction to eNOS.

Localization to the cell membrane may also facilitate rapid activation of Akt, which is responsible for estrogen-dependent eNOS activation. Indeed, PtdIns-3,4,5-P₃ production by PI3K depends on the local availability of lipid substrates, which is maximal at the cell membrane and is necessary for the activation of PDK-1 and -2. These 2 kinases mediate the serine and threonine phosphorylation of Akt.¹⁴ ¹⁵ We show that estrogen-dependent activation of Akt through PI3K is mediated through phosphorylation on these residues, suggesting that the localization of ERs to cell membrane and their recruitment of PI3K may create a microenvironment at this site that facilitates eNOS activation (which is prevalently localized to the cell membrane, as well) by activated Akt.

In conclusion, our experiments provide evidence for an important role of cell membrane–bound estrogen receptors for binding and activation of PI3K, which leads to eNOS activation. These findings suggest an important function for the extranuclear fraction of estrogen receptors in mediating rapid, nontranscriptional signaling of estrogen.

Acknowledgments

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References