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

Tommaso Simoncini, Elena Rabkin, James K. Liao

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;23:198-203.)

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 estrogen 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.19 Moreover, there is lack of information on the specific molecular mechanisms that link PI3K activation to 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 I A collagenase. MCF-7 cells were from ATCC, and p85α11 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 nitrite assay using 2,3-diaminonaphthalene, as described.8 Endothelial cells were harvested in PBS containing 1 mmol/L EDTA, and cell lysates were assayed for eNOS activity, as described.8

Immunoprecipitations and PI3K Assays

Endothelial cell protein extracts were immunoprecipitated 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.8 The labeled phospholipids were extracted with chloroform/methanol, and the organic phase, containing the PI3K products, was separated by borate TLC according to Walsh.20

Immunoblotting

Endothelial cell lysates were separated by SDS-PAGE, and immunoblottings 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), Gα (Santa Cruz, clone E-17), Sp1 (Santa Cruz, clone PEP 2), ERα (NeoMarkers, clone TE111), wild-type Akt (catalogue No. 65-558) and Thr308-P-Akt (catalogue No. 65-678) or Ser473-P-Akt (catalogue No. 65-801) (all from Upstate Biotechnology), eNOS (Transduction Laboratories, clone 3), inducible NOS (iNOS) (Transduction Laboratories, clone 6), wild-type ERK 1/2 (catalogue No. 442704), or Tyr368-P-ERK 1/2 (catalogue No. 442705) (Calbiochem).

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.

Results

In human endothelial cells, physiological concentrations of 17β-estradiol (E2) acutely increase NO release via an ER and PI3K-dependent mechanism.8 Different from insulin-dependent activation, type III NOS is recruited in a biphasic manner (EC50 value of ~0.1 mmol/L), showing an early induction within 2 minutes, followed by a more substantial increase after 15 to 20 minutes (Figure 1A). The initial increase is mediated by tyrosine kinases and MAP kinases,7 because it is partially prevented by genistein or by PD 98059 (WM) on E2-induced eNOS activation in HSVECs. *P<0.05 compared with time-corresponding E2 only–treated cells.

Cell Immunofluorescent Analysis

Endothelial cells were fixed with 3% paraformaldehyde. Cells were incubated overnight with anti-p85α antibody (Pharmingen, clone U15, 1:50 in 1% BSA) and mouse anti-ERα (NeoMarkers, clone TE111, 1:50 in 1% BSA) at 4°C. Immunofluorescent staining was then performed as described.21

Transfection Assays

The Akt constructs have been described previously.22 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.8

Figure 1. Acute activation of eNOS by estrogen: relative role of PI3K, Tyr kinases, and MAP kinases. A, Kinetics of eNOS activation in HSVECs by E2 (10 nmol/L) or insulin (100 nmol/L). B, Effects of Tyr kinases inhibitor genistein (GS) (50 µmol/L) or of MAP kinases inhibitor PD 98059 (PD) (5 µmol/L) versus wortmannin (WM) on E2-induced eNOS activation in HSVECs. *P<0.05 compared with time-corresponding E2 only–treated cells.
Figure 2. Mechanism of estrogen-stimulated eNOS and PI3K activation. A, HSVEC lysates were treated for 30 minutes with E2 (10 nmol/L) or with insulin (100 nmol/L), immunoprecipitated with either anti-ERα or anti-IRS-1 Abs (respectively), and then immunoblotted for the detection of associated p85α. B, HSVECs were treated for different times with E2 (10 nmol/L) or with insulin (100 nmol/L), immunoprecipitated with either anti-IRS-1 or 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 HSEVECs. D, Effect of insulin (100 nmol/L, 30 minutes) on basal or on estrogen-activated ERα-associated PI3K in HSEVECs.

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 observations, 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 cell membrane–associated Gcα and for the nuclear transcription factor Sp1. ERα is mainly nuclear, but there is a slight enhancement of the cytoplasmic/cell membrane distribution after E2 treatment (Figure 3C). p85α, instead, is mainly cytoplasmic, and its distribution does not change with exposure to estrogen (Figure 3C).

Because cell membrane ERs have been described in endothelial cells, and they have been involved in eNOS regulation,19 we studied whether the membrane-impermeable E2-BSA complex was able to activate eNOS. Compared with E2, E2-BSA is still able to activate E2, but the kinetics are slightly 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 phosphatidyl-dependent kinase (PDK)-1 and -2.14,15 Estrogen increases Akt kinase activity,8 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). 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 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

Figure 3. Cell membrane/cytoplasmic ERα is responsible for interaction with p85α. A and B, HSVGCS (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 HSVGCS 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 HSVGCS 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-estriadiol (E2, 10 nmol/L), 17α-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) in HSVGCS. 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.
whether Akt coprecipitates with them. Our results show that this is not the case (Figure IIIA), suggesting that Akt phosphorylation and eNOS activation take place as separate processes with 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,⁸ 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 kinaseinactive dominant-negative Akt mutant with a point mutation in the ATP binding domain, Akt (K179M), doesn’t 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.¹⁸ Since then, estrogen receptors localized on the cell membrane have been proposed to be involved with 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.²⁵

Several intracellular signaling cascades have been associated with rapid estrogen-dependent effects: the adenylyl cyclase pathway,²⁶ the phospholipase C pathway,²⁷ the G-protein–coupled receptor-activated pathways,²⁸ as well as the MAP kinase pathway.²⁹

At the vascular level, cell-surface ERs have been proposed to mediate estrogen-dependent rapid activation of NO synthesis,¹⁹,³⁰ and this seems to be partially dependent on MAP kinase activation.³⁰ 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.⁸

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.¹²,³¹,³²

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,²⁶ but still no data are available on possible conformational differences between these subpopulations. We have shown that ERα interacts with p85α after a conformational change dependent on estradiol binding and that this phenomenon does not require adapter molecules.³³ 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,³⁴ therefore inducing ER cycling on and off the nucleus, which may possibly serve to create a frequent sampling of the extracellular hormonal milieu.³⁵ 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,⁶ 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,³⁴ although ERβ (and not ERα) expression increases steeply after the injury,³⁵ 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 extracellular fraction of estrogen receptors in mediating rapid, nontranscriptional signaling of estrogen.

Acknowledgments

This work was supported by grants from the National Institutes of Health (HL48743, HL70274, and HL52233), the Ministero per l’Università e la Ricerca Scientifica e Tecnologica (MURST), and the University of Pisa. Dr Liao is an Established Investigator of the American Heart Association.

References

Molecular Basis of Cell Membrane Estrogen Receptor Interaction With Phosphatidylinositol 3-Kinase in Endothelial Cells
Tommaso Simoncini, Elena Rabkin and James K. Liao

Arterioscler Thromb Vasc Biol. 2003;23:198-203; originally published online December 26, 2002;
doi: 10.1161/01.ATV.000053846.71621.93
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/23/2/198

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2003/02/17/23.2.198.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Acute activation of eNOS by estrogen: relative role of PI3K, Tyr kinases and MAP kinases.
ERK-1/2 phosphorylation after E₂ (10 nM) treatment of HSVEC and effect of PD 98059 (PD) (5 µM).
Acute activation of eNOS by estrogen: relative role of PI3K, Tyr kinases and MAP kinases.

Effect of actinomycin D (ACT, 5 μM), 5,6-dichlorobenzimidazole riboside (DRB, 50 μM), and cycloheximide (CHX, 10 μM) on E₂ (10 nM)- or insulin (100 nM)-stimulated eNOS activity. HSVEC were pre-treated with ACT, DRB, or CHX for 2 hours prior to stimulation. The eNOS activity was measured 30 minutes after stimulation and standardized to control (no stimulation).

*indicates p<0.05 compared to control.
**Acute activation of eNOS by estrogen: relative role of PI3K, Tyr kinases and MAP kinases.**

Bovine aortic endothelial cells were transfected with vector or a luciferase reporter gene construct containing a tandem palindromic estrogen-response element (pERE) and luciferase activity was measured after E₂ (10 nM) stimulation for 30 minutes in the presence or absence of actinomycin D (ACT, 5 µM). Cells were co-transfected with pCMV.β-Gal and pre-treated with actinomycin D for 2 hours prior to E₂ stimulation. The pERE luciferase activity was standardized to the corresponding β-galactosidase activity and the values were normalized to that of vector transfection (fold induction). *indicates p<0.01 compared to transfection with pERE.
Mechanism of Estrogen-stimulated eNOS and PI3K activation.
HSVEC were treated for different times with $E_2$ (10 nM) in the presence or absence of 100 nM tamoxifen (TM) and cell lysates were immunoprecipitated with a monoclonal Ab vs. p85α ad then used for immunoblotting with ERα Ab.
Mechanism of Estrogen-stimulated eNOS and PI3K activation. HSVEC lysates were immunoprecipitated with a specific progesterone receptor (PR) Ab and then Western analysis was performed with either an anti-PR or an anti-p85α Ab.
Mechanism of Estrogen-stimulated eNOS and PI3K activation.
Relative ratio between ERα-recruited PI3K and total cellular PI3K and their responsiveness to estrogen treatment in HSVEC.
**Mechanism of Estrogen-stimulated eNOS and PI3K activation.**
Effect of ICI 182,780 (ICI, 10 µM), 5,6-dichlorobenzimidazole riboside (DRB, 50 µM) and cycloheximide (CHX, 10 µM) on ERα-associated PI3K activity in HSVEC. Inhibitors were added 2 h before E2 stimulation.
Activation of Akt through Ser/Thr phosphorylation mediates estrogen-induced eNOS activation. Lack of co-immunoprecipitation of Akt, ERα and p85α in HSVEC.
Activation of Akt through Ser/Thr phosphorylation mediates estrogen-induced eNOS activation.

Absence of co-immunoprecipitation of eNOS, ERα and p85α in HSVEC.
Akt accounts for eNOS activation by estrogen in endothelial cells.
Effect of E$_2$ (10 nM, 30 min) on eNOS activity in bovine aortic endothelial cells (BAEC) and murine fibroblasts (FB p85$\alpha$ +/-) transfected with empty vector (control), wild-type Akt (WT), constitutively-active Akt mutants (myr and ΔPH-myr), or a dominant-negative Akt mutant (K179M). The fibroblasts were also co-transfected with ER$\alpha$ and eNOS and all cells were co-transfected with pCMV.β-Gal. The eNOS activity was standardized to the corresponding β-galactosidase activity and the values were normalized to that of control. *indicates p<0.05 compared to control and **indicates p<0.05 compared to that of E$_2$ stimulation. Results shown are from three separate experiments performed in duplicates.
Detailed Methods (Online)

Cell cultures

Human saphenous and bovine aortic endothelial cells were harvested with Type IA collagenase (1 mg/mL) as described 1. Endothelial cells were maintained in phenol red-free Medium 199 (Gibco BRL, Life Technologies, Gaithersburg, MD), containing HEPES (25 mmol/L), heparin (50 U/mL), endothelial cell growth factor (ECGF) (50 µg/mL), L-glutamine (2 mmol/L), antibiotics, and 5% estrogen-deprived fetal bovine serum. Fetal bovine serum was deprived of estrogen by activated charcoal-stripping. Once grown to confluence the cells were replated on 1.5% gelatin-coated flasks at 20000 cells/cm2. HSVEC or PAEC isolated by this technique form a confluent monolayer of polygonal cells and express von Willebrand factor as determined by their content of immunoreactive protein. HSVEC were used for all experiments excluding transfections, where BAEC were used. MCF-7 cells were from American Tissue Type Collection, and p85α positive mice fibroblasts were a gift of Dr. L. Cantley (Boston, USA). MCF-7 and p85α positive mice fibroblasts were grown in DMEM (Gibco BRL) with standard nutrients. Cell number was assessed by direct cell counting of adherent cells, after trypsin detachment, in a Neubauer hemocytometer (VWR Scientifcs). The percentage of cells excluding Trypan Blue after staining with the compound was taken as a measure of cell viability. All cells were stimulated under serum-starved conditions consisting of phenol red-free Medium 199 or DMEM with 0.4% charcoal-stripped fetal calf serum.
Nitrite assay

NO accumulation from serum-starved endothelial cells was determined by a modified nitrite assay using 2,3-diaminonaphthalene, as described \(^2\). Briefly, fluorescence of 1-(H)-naphtotriazole was measured with excitation and emission wavelengths of 365 and 450 nm, respectively. Standard curves were constructed with known amounts of sodium nitrite. Nonspecific fluorescence was determined in the presence of LNMA (3 mM).

eNOS activity assay

Endothelial cells were harvested in PBS containing 1 mM EDTA, and cell lysates were assayed for eNOS activity as described \(^2\). HSVEC were grown to confluence in 55 cm\(^2\) culture dishes, treated accordingly with the experimental protocol, and harvested in ice-cold PBS containing 1 mM EDTA. Cell lysates were pelleted in a microfuge (2 minutes, 13000 rpm, 4°C) and subsequently homogenized in a buffer containing 25 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA. Endothelial nitric oxide synthase activity was detected by measuring the conversion of [3H]L-arginine to [3H]L-citrulline with the nitric oxide synthase assay kit (Calbiochem, La Jolla, CA), according to the manufacturer’s instructions. Rat cerebellum extracts, containing elevated amounts of iNOS, were used as positive controls, while endothelial cell extracts incubated in the presence of the competitive NOS inhibitor L-NAME (1 mM) were used to subtract the nonspecific activity.
Immunoprecipitations

Endothelial cell lysates were prepared by harvesting cell monolayers with 20 mM Tris, pH 7.4, 10 mM EDTA, 100 mM NaCl, 0.5 % NP-40 and protease inhibitors. Equal amount of lysates were incubated with 1 µg of precipitating antibody for 1 hour at 4°C under gentle agitation. 25 µL of a 1:1 protein A-agarose slurry were added to the tubes, and the samples were rolled at 4°C for another hour. The samples were then pelleted and washed 5 times with 20 mM Tris, pH 7.4, 10 mM EDTA, 150 mM NaCl, 0.5 % NP-40 containing protease inhibitors. After the washes, the pellets were resuspended in 50 µL of 2X Laemmli buffer and boiled for 5 min. The supernatants were separated on a SDS-PAGE gel, transferred to a nylon membrane and immunoblotted.

PI3K assays

PI3 kinase activity in endothelial cell lysates was assayed using the borate thin layer chromatography method, as described 3. Following a 6-hour starvation in medium 199 containing 0.4% estrogen deprived-FBS, cells were treated according to the experimental protocol, washed and harvested in ice-cold lysis buffer (137 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1 mM CaCl₂, 1 mM MgCl₂, 0.1 mM Na₃VO₄, 1% NP-40). After pelleting the cell debris, the supernatant was incubated for 1 hour at 4°C with 1 µg of a specific precipitating antibody. Protein/Ab complexes were subsequently immunoprecipitated with the addition of 25 µL of a 1:1 slurry of protein A-agarose for 1 hour at 4°C and subsequent centrifugation. The immunoprecipitates were washed three times with lysis buffer, three times with 0.1 M Tris-HCl, pH 7.4, 5 mM LiCl, 0.1 mM Na₃VO₄, followed by two washes
with 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1 mM Na3VO4. The immunoprecipitates were then mixed with 50 µL Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 20 µg of PtdIns-4,5-P2 (Sigma, Saint Louis, MO), 10 µL 100 mM MgCl2 and 5 µL of a 0.88 mM ATP, 20 mM MgCl2 solution, containing 30 µCi of [γ-32P]ATP (3000 Ci/mmol; NEN Life Science Products, Boston, MA). The reaction was incubated at 37°C for 10 minutes, and subsequently blocked by the addition of 20 µL of 6N HCl. The phospholipids were extracted with 160 µL of chloroform/methanol (1:1, v/v). 50 µL of the organic phase, containing the labeled PI3 kinase products were separated by borate thin layer chromatography on glass-backed Silica Gel 60 plates (EM Separations, Gibbstown, NJ) pretreated with a solution containing 25 mM trans-1,2-diaminocyclohexane-N,N,N’N’-tetraacetic acid (CDTA, Sigma, Saint Louis, MO), 66% (v/v) ethanol and 0.06 N NaOH, dried for 1 hour and then baked at 100°C for 15 minutes. The chromatography was developed with a solution containing 37.5% (v/v) methanol, 30% (v/v) chloroform, 22.5% (v/v) pyridine (Sigma, Saint Louis, MO), 1.33% (v/v) formic acid, 1 M boric acid and 8.5 mM butylated hydroxitoluene (Sigma, Saint Louis, MO), briefly dried and exposed to autoradiography.

Immunoblotting
After the different treatments, endothelial cells were rinsed once with ice-cold PBS before addition of the lysis buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 1 mM sodium orthovanadate, 1 mM NaF, and 1 mM phenylmethylsulfonylfluoride) to the dishes on an ice tray. The cell lysates were scraped, boiled, and centrifuged for 2 min at 13000
rpm. Total cell lysates (40 µg of protein) and low range molecular weight markers (BIO-RAD, Hercules, CA) were separated by SDS-polyacrylamide gel electrophoresis (12% running, 4% stacking) and electrophoretically transferred to polyvinylidene fluoride membranes (Immobilon P, 0.45 µm pore size) and incubated overnight at 4°C with blocking solution (5% skim milk in TBS - 0.1% Tween 20). Affinity-purified rabbit antibodies (0.4 µg of IgG/ml) were incubated with the blots overnight at 4 °C in TBS - 0.1% Tween 20 containing 5% BSA. The blots were washed three times with TBS - 0.1% Tween 20 and then treated with specific secondary antibodies (1:2000 dilution) coupled to horseradish peroxidase. Immunodetection was accomplished using the enhanced chemoluminescence kit (ECL Kit, Amersham Corp).

Cell immunofluorescent analysis

Endothelial cells were grown on gelatin-coated coverslips in 6-well plates. After treatments, endothelial cells were fixed and permeabilized with 3% paraformaldehyde. Blocking was performed with 3% normal goat serum for 20 min. Cells were incubated overnight with anti-p85α antibody (1:50 in 1% BSA) and mouse anti-ERα (1:50 in 1% BSA) at 4 °C. Immunofluorescent staining was then performed as described 4. Briefly, a biotinylated secondary antibody was used. After 45 min of incubation with the secondary antibody, streptavidin-fluorescein isothiocyanate was added for 45 min. Immunofluorescence was visualized using an Olympus BX 60F microscope. Photographic images were taken from different random fields.
Transfection assays

The wild type as well as the mutant Akt constructs have been described previously. BAEC and murine wild type fibroblasts were transfected with each plasmid (4 µg) using the Lipofectamine reagent system (Gibco BRL). As an internal control for transfection efficiency, pCMV-β-Gal plasmid (2 µg), containing the β-galactosidase gene was cotransfected in all experiments. β-galactosidase staining indicated that cellular transfection efficiency was approximately 30-35%. Cells (60-70% confluent) were harvested and cell extracts were prepared using lysis buffer (100 µg/ml leupeptin, 50 µg/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, 5 mM EGTA, 100 mM NaCl, 5 mM Tris-HCl, pH 7.4), as described. Briefly, cell extracts were centrifuged at 12,000 x g for 10 min, and the supernatant was used for eNOS activity and β-galactosidase assays. The relative eNOS activity was calculated as the ratio of eNOS to β-galactosidase activity.

β-galactosidase assays

β-Galactosidase activity was assayed spectrophometrically (absorption at 410 nm) and compared to a standard curve using known amounts of purified β-galactosidase (Sigma) as described previously.

Statistical analysis

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


