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Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase

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Abstract

Oestrogen produces diverse biological effects through binding to the oestrogen receptor (ER)¹. The ER is a steroid hormone nuclear receptor, which, when bound to oestrogen, modulates the transcriptional activity of target genes². Controversy exists, however, concerning whether ER has a role outside the nucleus³, particularly in mediating the cardiovascular protective effects of oestrogen⁴. Here we show that the ER isoform, ER α , binds in a ligand-dependent manner to the p85 α regulatory subunit of phosphatidylinositol-3-OH kinase (PI(3)K). Stimulation with oestrogen increases ER α -associated PI(3)K activity, leading to the activation of protein kinase B/Akt and endothelial nitric oxide synthase (eNOS). Recruitment and activation of PI(3)K by ligand-bound ER α are independent of gene transcription, do not involve phosphotyrosine adapter molecules or *src*-homology domains of p85 α , and extend to other steroid hormone receptors. Mice treated with oestrogen show increased eNOS activity and decreased vascular leukocyte accumulation after ischaemia and reperfusion injury. This vascular protective effect of oestrogen was abolished in the presence of PI(3)K or eNOS inhibitors. Our findings define a physiologically important non-nuclear oestrogen-signalling pathway involving the direct interaction of ER α with PI(3)K.

PI(3)K mediates the cellular effects of platelet-derived growth factor (PDGF)⁵, insulin⁶ and vascular endothelial growth factor (VEGF)⁷. The predominant form of PI(3)K comprises p85 α , an adapter/regulatory subunit of relative molecular mass 85,000 (M_r 85K), and p110, a catalytic subunit⁸ of M_r 110K. PI(3)K catalyses the formation of lipid mediators^{9,10} which recruit signalling molecules containing phosphatidylinositol (PtdIns)-3,4,5-P₃-binding or pleck-strin homology domains such as phosphatidylinositol-dependent kinases and protein kinase Akt^{11,12}. The activation of Akt through phosphorylation of Thr 308/Ser 473 (ref. 13) mediates many of the downstream cellular effects of PI(3)K, including stimulation of glucose transporter-4 membrane translocation¹⁴, inactivation of glycogen synthase kinase-3 (ref. 15), and activation of eNOS^{16,17} and cell survival pathways¹⁸. Although oestrogen stimulates

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eNOS activity¹⁹ and promotes cell survival, it is not known whether PI(3)K mediates these effects of oestrogen.

In human vascular endothelial cells, physiological concentrations of 17 β -oestradiol (E₂) increased eNOS activity in a biphasic manner (effector concentration for half-maximal response (EC₅₀) $\approx$ 0.1 nM) (Fig. 1a, b). The initial increase was mediated by mitogen-activated protein (MAP) kinases¹⁹; the second increase was completely blocked by the PI(3)K inhibitor, wortmannin. The increase in eNOS activity was also blocked by the ER antagonist ICI 182,780; and the inactive E₂ stereoisomer 17 α -oestradiol (α E₂) had no effect. In murine fibroblasts transfected with ER α and eNOS complementary DNAs, E₂ produced an eightfold increase in eNOS activity in wild-type but not in p85 α -deficient (p85 α ^{-/-}) fibroblasts²⁰ (Fig. 1c). Furthermore, in p85 α ^{-/-} fibroblasts co-transfection of p85 α cDNA led to a fourfold increase in E₂-stimulated eNOS activity, whereas in wild-type fibroblasts co-transfection of a dominant-negative p85 α mutant cDNA decreased E₂-stimulated eNOS activity by more than 50%.

In non-transfected human endothelial cells, E₂ increased endogenous PtdIns-3,4,5-P₃ levels in a time-delayed manner similar to the wortmannin-sensitive phase of eNOS activation (Fig. 2a). In contrast, insulin rapidly increased endogenous PtdIns-3,4,5-P₃ levels⁶ and eNOS activity²¹. Increases in PtdIns-3,4,5-P₃ levels correlated temporally with the ligand-dependent increases in ER α -associated PI(3)K activity (Fig. 2b); events that were blocked by ICI 182,780 and wortmannin (Fig. 2c). Consistent with a rapid, non-nuclear effect of ER on eNOS activation, E₂-stimulated PI(3)K activity was blocked by another ER antagonist, tamoxifen, but not by the MAP kinase inhibitor PD 98059, or by the transcriptional inhibitor actinomycin D (Fig. 2d). Insulin, which uses the phosphotyrosine (p-Tyr) adapter molecule, insulin receptor substrate (IRS)-1, to interact with PI(3)K, increased PI(3)K activity in the p-Tyr and IRS-1 immunoprecipitate (Fig. 2e), but did not increase or augment E₂-associated PI(3)K activity. In contrast, E₂ did not increase p-Tyr- or IRS-1-associated PI(3)K activity (Fig. 2e). These findings suggest that ER α does not recruit PI(3)K that has been already activated by insulin, and that PI(3)K activation by ER and IRS-1 occurs through different mechanisms. Notably, the activation of PI(3)K extended to other steroid hormone nuclear receptors such as the thyroid hormone and glucocorticoid receptors (Fig. 2f). These interactions may explain some of the previously unrecognized functions of these nuclear hormone receptors.

ER α interacted with p85 α in a ligand-dependent manner in both non-transfected endothelial cells (Fig. 3a) and p85 α ^{-/-} fibroblasts transfected with ER α and p85 α cDNAs (Fig. 3b). This ligand-dependent interaction was blocked by ICI 182,780 and was absent in p85 α ^{-/-} fibroblasts transfected with ER α cDNA alone. However, the ER isoform ER β , which is thought to mediate some of the cardiovascular effects of oestrogen⁴, did not interact with p85 α or recruit PI(3)K activity after E₂ stimulation (see Supplementary Information). The interaction of ER α and p85 α also occurred in the absence of adapter molecules or accessory proteins, as human recombinant ER α could still interact with glutathione *S*-transferase (GST)—p85 α fusion protein in a ligand-dependent manner in a cell-free system (Fig. 3c). This interaction, however, does not involve the *src*-homology SH2/SH3 domains of p85 α (Fig. 3d) which interact with p-Tyr residues of growth hormone receptors and adapter molecules^{22,23}. Heat shock protein 90, which binds and facilitates the function of ER²⁴ and eNOS²⁵, inhibited the interaction of ER α and p85 α .

The generation of PtdIns-3,4,5-P₃ leads to the recruitment and activation of Akt^{11,26}. E₂ stimulated Akt kinase activity in a time-delayed manner (Fig. 3e), similar to the increases observed in PtdIns-3,4,5-P₃ levels and eNOS activity. To determine whether E₂-stimulated eNOS activation is mediated by Akt, we transiently transfected bovine aortic endothelial cells with adenoviruses containing constitutively active (myr) and dominant-negative (dn) Akt mutants²⁷. Transfection of these cells with myr-Akt produced a substantial increase in eNOS

activity, whereas overexpression of dn-Akt decreased basal eNOS activity below baseline and completely abolished E₂-stimulated eNOS activity (Fig. 3f).

To determine the physiological significance of this pathway, we used an established model of ischaemia and reperfusion (I/R) injury in the mouse cremaster muscle²⁸. I/R leads to leukocyte recruitment to the vascular wall, an event attenuated by NO and exacerbated by eNOS inhibitors such as L-nitroarginine methylester (L-NAME)²⁹. I/R reduced median leukocyte rolling velocity by 13.8 μms^{-1} ($P < 0.003$) and induced a 2.2-fold increase in the number of adherent leukocytes ($P < 0.001$) (Fig. 4a, b). Treatment with E₂ increased eNOS activity 3.2-fold and prevented the subsequent changes in leukocyte accumulation and rolling velocity after I/R. When wortmannin or L-NAME was applied to the cremaster muscle, measurements of leukocyte rolling velocity and accumulation were not different between untreated and E₂-treated mice after I/R, although L-NAME decreased eNOS activity below that of untreated mice (Fig. 4a—c). These findings indicate that the NO-induced vascular protective effect of oestrogen is predominantly mediated by PI(3)K.

Although the nuclear function of ER is clearly established, previous studies regarding the membrane and cytoplasmic effects of oestrogen remain inconclusive³. Linking the ER to PI(3)K suggests that the ER may be involved in a critical function outside the nucleus. In addition, the potential biological effects of oestrogen are considerably broadened because PI(3)K is known to mediate various cellular functions¹⁸. Although most of the ER is localized to the nucleus, we found that there is an increased level of membrane and cytoplasmic ER after E₂ stimulation (data not shown). Indeed, a study has suggested that membrane-associated ER is involved in mediating NO release from endothelial cells³⁰. Thus, it is likely that PI(3)K is being recruited and activated by a small subset of ligand-bound, membrane-associated ERs. It remains to be determined, however, whether oestrogen can also activate PI(3)K indirectly, and whether PI(3)K can account for other rapid, non-nuclear effects of oestrogen. Further studies characterizing the interaction domains of ER α and p85 α should help clarify these issues.

Methods

Cell cultures

Human and bovine aortic endothelial cells were obtained enzymatically with Type IA collagenase (1 mg ml⁻¹). They were cultured and stimulated under serum-starved conditions consisting of phenol-red-free Medium 199 (Gibco BRL, Life Technologies) with 0.4% charcoal-stripped fetal calf serum.

Immunoprecipitations

Cells were washed with ice-cold PBS and lysed with the following buffer: Tris-HCl (20 mM, pH 7.4), EDTA (10 mM), NaCl (100 mM), IGEPAL (1%), Na₃VO₄ (1 mM), NaF (50 mM), PMSF (0.1 mg ml⁻¹) and aprotinin (0.3 mg ml⁻¹). We added the immunoprecipitating antibody (1 μg) to equal amounts of cell lysates (0.5–1 mg) in 500 μl of lysis buffer for 1 h at 4 °C with gentle rocking. Afterwards, 40 μl of 1:1 Protein-A-agarose was added and the entire mixture was rocked gently for another 1 h at 4 °C. The mixture was then centrifuged at 12,000g for 5 min at 4 °C. The supernatant was removed and the immunoprecipitate was washed three times with 500 μl of washing buffer, which differs from the lysis buffer in having 150 mM NaCl instead of 100 mM NaCl. We then separated proteins in the washed immunoprecipitate by SDS-PAGE and immunoblotted them with anti-ER α (Ab-10: Clone TE111.5D11, NeoMarkers, Fremont, CA) or anti-p85 α (Upstate Biotech., Lake Placid, NY) antibody.

GST fusion protein-affinity purification

Human recombinant GST—p85 α fusion protein or GST (Sigma) bound to glutathioneagarose beads (1 μ g protein per 20 μ l beads) was suspended in 400 μ l of *Escherichia coli* protein extract solution (10 mg ml⁻¹) and incubated with 1 μ g human recombinant ER α (Panvera, Madison, WI) for 1 h at 4 °C. We pelleted the samples, and washed the beads five times with a buffer containing 50 mM potassium phosphate, pH 7.5, 150 mM KCl, 1 mM MgCl₂, 10 % (v/v) glycerol and 1% (v/v) Triton X-100 plus protease inhibitors. The beads were re-suspended in 50 μ l of 2 $\times$ Laemmli's buffer and boiled for 5 min. Proteins were separated on SDS-PAGE.

Model of vascular injury

Ten-week-old, 24 g, male C57BL/6 mice (Hilltop, Scottsdale, PA) were subcutaneously implanted with 1.5 mg of slow-release E₂ tablets (Innovative Research of America, Sarasota, FL) 3–5 days before experiments to ensure steady-state serum E₂ levels and to avoid any effects of surgery on baseline haemodynamic parameters. Mice implanted with E₂ tablets had a serum E₂ level of 760 $\pm$ 30 pg ml⁻¹ compared with that of vehicle-treated mice (24 $\pm$ 6 pgml⁻¹). Mice were anaesthetized and the cremaster muscle was studied under intravital microscopy²⁸. Ischaemia was induced by applying pressure to supplying arteries just sufficient to stop blood flow for 30 min. In some experiments, wortmannin (100 nM) or L-NAME (0.1 mM) was applied to the cremaster muscle during the ischaemic period. The pressure was released for reperfusion, and the same vessels were recorded in each animal before and after I/R. The rolling velocities of 25 leukocytes were measured in each venule, sorted and averaged for each rank to construct cumulative histograms. The velocities of 3,750 leukocytes were measured in 150 venules before and after I/R. The number of firmly adherent leukocytes was measured before and after I/R in the same 200- μ m long segments of venules. The following number of venules were studied for leukocyte adhesion: untreated, 15 venules; E₂-treated, 20 venules; E₂-treated with wortmannin, 25 venules; E₂-treated with L-NAME, 15 venules. Cremaster eNOS activity was measured in three untreated, four E₂-treated, five E₂-treated with wortmannin and four E₂-treated with L-NAME mice.

Supplementary Material

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References

1. Green S, et al. Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. *Nature* 1986;320:134–139. [PubMed: 3754034]
2. Kumar V, et al. Functional domains of the human estrogen receptor. *Cell* 1987;51:941–951. [PubMed: 3690665]
3. Pietras RJ, Szego CM. Specific binding sites for oestrogen at the outer surfaces of isolated endometrial cells. *Nature* 1977;265:69–72. [PubMed: 834244]
4. Gustafsson JA. Novel aspects of estrogen action. *J. Soc. Gynecol. Investig* 2000;7:S8–9.
5. Escobedo JA, et al. cDNA cloning of a novel 85 kd protein that has SH2 domains and regulates binding of PI3-kinase to the PDGF beta-receptor. *Cell* 1991;65:75–82. [PubMed: 1849460]

6. Ruderman NB, Kapeller R, White MF, Cantley LC. Activation of phosphatidylinositol 3-kinase by insulin. *Proc. Natl Acad. Sci. USA* 1990;87:1411–1415. [PubMed: 2154747]
7. Papapetropoulos A, Garcia-Cardena G, Madri JA, Sessa WC. Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. *J. Clin. Invest* 1997;100:3131–3139. [PubMed: 9399960]
8. Carpenter CL, et al. Purification and characterization of phosphoinositide 3-kinase from rat liver. *J. Biol. Chem* 1990;265:19704–19711. [PubMed: 2174051]
9. Auger KR, Serunian LA, Soltoff SP, Libby P, Cantley LC. PDGF-dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells. *Cell* 1989;57:167–175. [PubMed: 2467744]
10. Rameh LE, Cantley LC. The role of phosphoinositide 3-kinase lipid products in cell function. *J. Biol. Chem* 1999;274:8347–8350. [PubMed: 10085060]
11. Stephens L, et al. Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-trisphosphate-dependent activation of protein kinase B. *Science* 1998;279:710–714. [PubMed: 9445477]
12. Delcommenne M, et al. Phosphoinositide-3-OH kinase-dependent regulation of glycogen synthase kinase 3 and protein kinase B/AKT by the integrin-linked kinase. *Proc. Natl Acad. Sci. USA* 1998;95:11211–11216. [PubMed: 9736715]
13. Alessi DR, et al. Mechanism of activation of protein kinase B by insulin and IGF 1. *EMBO J* 1996;15:6541–6551. [PubMed: 8978681]
14. Kohn AD, Summers SA, Birnbaum MJ, Roth RA. Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. *J. Biol. Chem* 1996;271:31372–31378. [PubMed: 8940145]
15. Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 1995;378:785–789. [PubMed: 8524413]
16. Dimmeler S, et al. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* 1999;399:601–605. [PubMed: 10376603]
17. Fulton D, et al. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* 1999;399:597–601. [PubMed: 10376602]
18. Franke TF, Kaplan DR, Cantley LC. PI(3)K: downstream AKTion blocks apoptosis. *Cell* 1997;88:435–437. [PubMed: 9038334]
19. Chen Z, et al. Estrogen receptor alpha mediates the nongenomic activation of endothelial nitric oxide synthase by estrogen. *J. Clin. Invest* 1999;103:401–406. [PubMed: 9927501]
20. Fruman DA, et al. Impaired B cell development and proliferation in absence of phosphoinositide 3-kinase p85 α . *Science* 1999;283:393–397. [PubMed: 9888855]
21. Zeng G, Quon MJ. Insulin-stimulated production of nitric oxide is inhibited by wortmannin. Direct measurement in vascular endothelial cells. *J. Clin. Invest* 1996;98:894–898. [PubMed: 8770859]
22. Fantl WJ, et al. Distinct phosphotyrosines on a growth factor receptor bind to specific molecules that mediate different signaling pathways. *Cell* 1992;69:413–423. [PubMed: 1374684]
23. Kapeller R, Toker A, Cantley LC, Carpenter CL. Phosphoinositide 3-kinase binds constitutively to α/β -tubulin and binds to gamma-tubulin in response to insulin. *J. Biol. Chem* 1995;270:25985–25991. [PubMed: 7592789]
24. Picard D, et al. Reduced levels of hsp90 compromise steroid receptor action *in vivo*. *Nature* 1990;348:166–168. [PubMed: 2234079]
25. Garcia-Cardena G, et al. Dynamic activation of endothelial nitric oxide synthase by Hsp90. *Nature* 1998;392:821–824. [PubMed: 9580552]
26. Burgering BM, Coffey PJ. Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature* 1995;376:599–602. [PubMed: 7637810]
27. Fujio Y, Walsh K. Akt mediates cytoprotection of endothelial cells by vascular endothelial growth factor in an anchorage-dependent manner. *J. Biol. Chem* 1999;274:16349–16354. [PubMed: 10347193]
28. Kanwar S, Smith CW, Kubes P. An absolute requirement for P-selectin in ischemia/reperfusion-induced leukocyte recruitment in cremaster muscle. *Microcirculation* 1998;5:281–287. [PubMed: 9866119]

29. Kubes P, Suzuki M, Granger DN. Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc. Natl Acad. Sci. USA* 1991;88:4651–4655. [PubMed: 1675786]
30. Stefano GB, et al. Cell-surface estrogen receptors mediate calcium-dependent nitric oxide release in human endothelia. *Circulation* 2000;101:1594–1597. [PubMed: 10747354]

Figure 1.

Activation of eNOS by oestrogen is mediated by PI(3)K. **a, b**, Concentration-dependent (**a**) and time-dependent (**b**) effects of E_2 and wortmannin (WM, 30 nM) on eNOS activity (fold induction versus baseline) in human vascular endothelial cells. Asterisk indicates $P < 0.05$ compared with unstimulated or E_2 stimulation. **c**, E_2 -stimulated NOS activity in murine p85 $\alpha^{+/+}$ and p85 $\alpha^{-/-}$ fibroblasts (FB) transfected (Tx) with vector (pcDNA3), eNOS, ER α , p85 α or dominant-negative p85 α (Δ p85 α) cDNAs. Asterisk indicates $P < 0.05$ compared with transfection with eNOS cDNA alone; two asterisks indicate $P < 0.05$ compared with transfection with ER α and eNOS cDNAs.

Figure 2.

Oestrogen stimulates ERα-associated PI(3)K activity. **a**, Effect of vehicle (ethanol 0.01% v/v), E₂ (10 nM) or insulin (100 nM) on endogenous PtdIns-3,4,5-P₃ levels. Asterisk indicates $P < 0.05$ compared with vehicle. **b**, Time-dependent effect of E₂ on ERα, p85α and PI(3)K activity (PIP₃) in ERα immunoprecipitate (IP). **c**, Effect of ICI (10 μM) or WM on E₂ or 17α-oestradiol (αE₂)-stimulated ERα-associated PI(3)K activity. Cells were pre-treated with ICI or WM for 30 min. **d**, Effect of tamoxifen (TM, 1 μM), PD 98059 (PD, 5 μM) and actinomycin D (ACT, 5 μM) on ERα-associated PI(3)K activity. Inhibitors were added 2 h before E₂ stimulation. **e**, Effect of E₂ or insulin (Ins) on p-Tyr- and IRS-1-associated PI(3)K activity. **f**, Effect of E₂, progesterone (Prog, 10 nM), testosterone (Test, 10 nM), thyroid hormone (Thyr,

10 nM), dexamethasone (Dex, 1 μ M), WY14643 (WY, 100 μ M) and 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (PGJ₂, 100 μ M) on PI(3)K activity in the corresponding steroid hormone nuclear receptor immunoprecipitates.

Figure 3.

Ligand-dependent interaction of ERα with p85α. **a, b**, Effect of E₂ on ERα—p85α co-immunoprecipitation in non-transfected human endothelial cells (**a**) and murine p85α^{-/-} fibroblasts (**b**) transfected (Tx) with ERα and p85α, alone or in combination. **c, d**, Affinity purification using agarose-conjugated GST or GST—p85α (**c**), or GST—p85α amino-terminal SH2 domain (NSH2, amino acids 321–470), carboxy-terminal SH2 domain (CSH2, 576–724), or SH3 domain (NSH3, 1–80) fusion protein and human recombinant (hr) ERα (**d**). **e**, E₂- or insulin (Ins)-stimulated Akt kinase activity. Asterisk indicates *P* < 0.05 compared with no stimulation. **f**, Effect of E₂ on eNOS activity (fold induction over baseline) in endothelial cells

transfected with adenovirus containing no Akt (vector), constitutively active (myr), or a dominant-negative (dn) Akt. Asterisk indicates $P < 0.05$ compared with vector alone.

Figure 4.

PI(3)K and NO mediate the vascular protective effects of oestrogen. Cumulative histograms of leukocyte rolling velocities before (-) and after (+) ischaemia and reperfusion (I/R) are shown. **a, b**, Effect of superfused WM (100 nM) or L-nitroarginine methylester (L-NAME, 0.1 mM) on leukocyte rolling velocity (**a**) and leukocyte adhesion and eNOS activity in the murine cremaster muscle (**b**). Data are expressed as fold increase over baseline before I/R in the same paired venules. Asterisk indicates $P < 0.001$ compared with untreated after I/R (None). **c**, Representative video images showing the same venules before (-) and after (+) I/R with the indicated treatments. Scale bar, 40 μm .