

Variant estrogen receptor–c-Src molecular interdependence and c-Src structural requirements for endothelial NO synthase activation

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Edited by Salvador Moncada, University of London, London, United Kingdom, and approved August 28, 2007 (received for review May 8, 2007)

Little is known about the tyrosine kinase c-Src's function in the systemic circulation, in particular its role in arterial responses to hormonal stimuli. In human aortic and venous endothelial cells, c-Src is indispensable for 17 β -estradiol (E2)-stimulated phosphatidylinositol 3-kinase/Akt/endothelial NO synthase (eNOS) pathway activation, a possible mechanism in E2-mediated vascular protection. Here we show that c-Src supports basal and E2-stimulated NO production and is required for E2-induced vasorelaxation in murine aortas. Only membrane c-Src is structurally and functionally involved in E2-induced eNOS activation. Independent of c-Src kinase activity, c-Src is associated with an N-terminally truncated estrogen receptor α variant (ER46) and eNOS in the plasma membrane through its "open" (substrate-accessible) conformation. In the presence of E2, c-Src kinase is activated by membrane ER46 and in turn phosphorylates ER46 for subsequent ER46 and c-Src membrane recruitment, the assembly of an eNOS-centered membrane macrocomplex, and membrane-initiated eNOS activation. Overall, these results provide insights into a critical role for the tyrosine kinase c-Src in estrogen-stimulated arterial responses, and in membrane-initiated rapid signal transduction, for which obligate complex assembly and localization require the c-Src substrate-accessible structure.

endothelium | eNOS

The most biologically potent, endogenous estrogen, 17 β -estradiol (E2), is able to enhance vascular NO bioavailability by chronically increasing endothelial NO synthase (eNOS) expression and acutely stimulating eNOS activity through estrogen receptor (ER)-dependent signal transduction, most notably involving the phosphatidylinositol 3-kinase/Akt/eNOS pathway in endothelial cells (EC). This notion serves as one fundamental explanation for E2-induced vascular protection against ischemia/reperfusion injury, atherosclerosis progression, and neointima formation. Although membrane ER α and membrane ER46 (a *de novo* truncated ER α) are now appreciated to initiate estrogen-stimulated, c-Src-dependent eNOS activation after acquiring c-Src activity (1–3), little is known about the molecular details of c-Src involvement and the importance of c-Src in estrogen-induced vasorelaxation.

In mammals, Src family kinases have at least eight structurally distinct members and pivotally regulate cell migration, adhesion, proliferation, differentiation, and survival (4). Among eight Src kinase family members, only c-Src, Fyn, and Yes are ubiquitously expressed. Phosphorylation of c-Src Y416 with concomitant Y527 dephosphorylation is a consequence of c-Src activation and often leads to full kinase activity (4). c-Src family members are location-sensitive, membrane-associated, nonreceptor tyrosine kinases. c-Src kinase is reversibly coupled to the inner leaflet of the plasma membrane via a myristate moiety covalently attached to the G2 residue and a polybasic cluster embedded in the N terminus (5). All Src tyrosine kinases have a characteristic structure including single Src homology (SH) 3, SH2, and protein

tyrosine kinase domains. Cytosolic c-Src is often self-restrained to an inactive conformation by both intramolecular and intermolecular interactions between its SH2 domain and phospho-Y527 and between its SH3 domain and SH2-kinase linker domains. Disrupting the inhibitory conformation by Y527 dephosphorylation or protein binding to the SH2 or SH3 domains imparts c-Src with an open structure permissive for Y416 autophosphorylation and resultant kinase activation (6, 7).

c-Src kinase can phosphorylate ER α at Y537 in resting cells, and, in turn, the c-Src SH2 domain can directly bind to ER α phospho-Y537 (8, 9). The "open" conformation, normally prompted via Y527 dephosphorylation by protein tyrosine phosphatase (PTP) α , PTP γ , PTP1B, or SHP-2 (6, 7), governs molecular recognition of c-Src substrates and its binding partners and is necessary for the redistribution of c-Src from endosomes to focal adhesions (10). In this article we present the molecular dissection of c-Src membrane–ER46 interactions, including interdependence for membrane targeting. We also display, by use of aortas obtained from c-Src gene-deleted animals, the critical role of c-Src in estrogen-induced vascular responses.

Results

c-Src Requirement in Basal and E2-Induced Vasorelaxation. To determine whether c-Src plays a physiological role in E2-induced vasomotor responses, we analyzed the basal and E2-modulated vascular tone *ex vivo* by myography. E2 (10^{-6} to 3×10^{-5} M) induced 10–65% vasorelaxation of L-phenylephrine (PE)-precontracted control aortic rings and 4–22% in c-Src^{−/−} aortic rings (representative myographs shown in Fig. 1A). That is, E2-induced vasodilation was markedly blunted in aortic rings from c-Src gene-deleted mice. The observed relaxation was partially suppressed by *N* ω -nitro-L-arginine methyl ester hydrochloride (L-NAME) ($38 \pm 2.6\%$, E2 = 3×10^{-5} M, $n = 6$, $P < 0.05$) and ICI 182,780 ($66 \pm 3.2\%$, E2 = 10^{-5} M, $n = 5$, $P < 0.05$), indicating that E2-enhanced NO bioavailability, through ER signaling, is at least partially responsible for the observed vasodilation (Fig. 1B). Endothelial denudation abolished E2-

Author contributions: L.L., K.H., M.P.H., A.S., M.C., and J.R.B. designed research; L.L., K.H., K.H.K., M.P.H., P.M.B., and M.C. performed research; P.M.B., R.B., and W.C.S. contributed new reagents/analytic tools; L.L., K.H., K.H.K., M.P.H., A.S., M.C., R.B., W.C.S., and J.R.B. analyzed data; and L.L., K.H.K., A.S., M.C., and J.R.B. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Abbreviations: E2, 17 β -estradiol; EC, endothelial cell; ER, estrogen receptor; L-NAME, *N* ω -nitro-L-arginine methyl ester hydrochloride; eNOS, endothelial NO synthase; PE, L-phenylephrine; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; PTP, protein tyrosine phosphatase; SH, Src homology.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0704315104/DC1.

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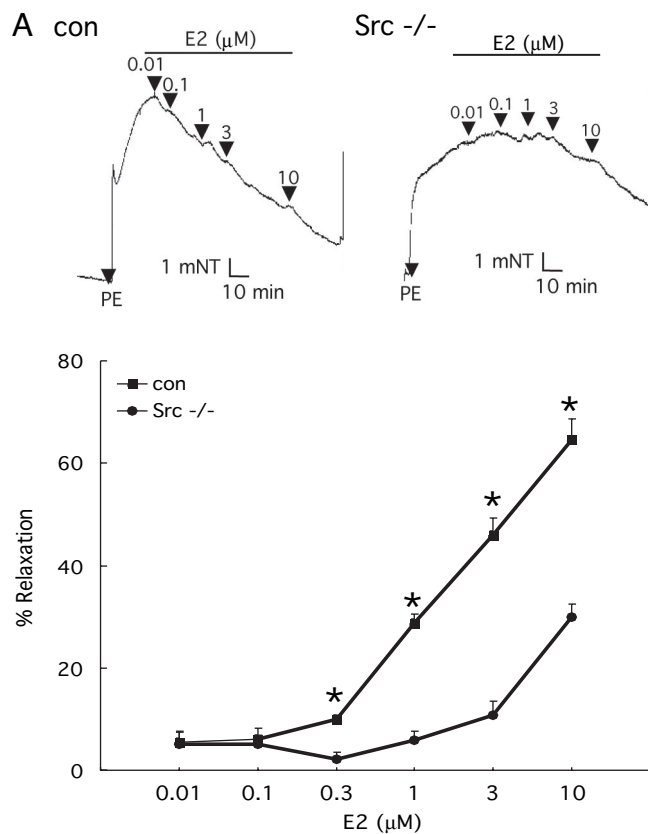


Fig. 1. c-Src-dependent basal and E2-induced vasorelaxation. (A) Impaired E2 induced vasodilation in PE-precontracted c-Src^{-/-} aortas. Control and Src^{-/-} thoracic aorta rings were isolated, precontracted with 10^{-6} M PE, and treated with E2, and the vascular tone was recorded by vessel myograph system. (B) Partially NO- and receptor-dependent vasorelaxation (10^{-8} M E2, 10^{-5} M ICI 182,780, and 10^{-4} M L-NAME). *, $P < 0.05$ (two-tailed ANOVA).

induced vasorelaxation in c-Src^{+/+} and c-Src^{-/-} aortic rings, and sodium nitroprusside (NO donor, 10^{-6} M) produced comparable vasodilating responses in both [see supporting information (SI) Fig. 7]. These results suggest that the differential response to E2 (Fig. 1A) was due largely to the difference in endothelium-derived vasodilator production and that smooth muscle responses were identical. In intact, non-denuded rings, acetylcholine (10^{-6} M) responses were also similar (see SI Fig. 7). This indicates not only that the endothelium is intact and eNOS responses are preserved in c-Src^{-/-} mice, but also that there is some specificity to the molecular complex that transduces

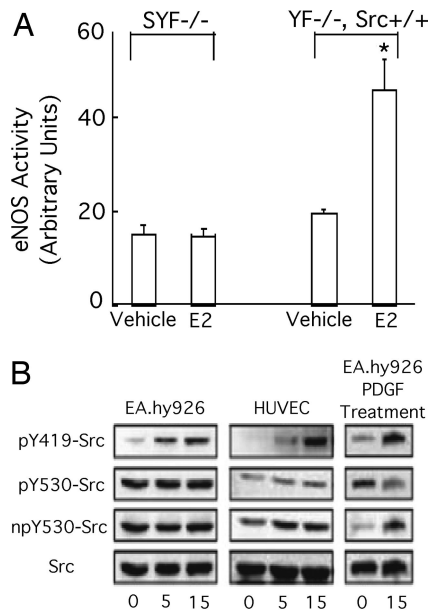


Fig. 2. Requirement of c-Src in ER46-mediated eNOS activation. (A) Activation of eNOS by E2 in reconstituted YF^{-/-}, Src^{+/+} but not SYF^{-/-} cells. Cells were transfected with eNOS and ER46, challenged with 30 nM E2 for 10 min, and subjected to eNOS activity assay. *, $P < 0.05$, $n = 4$. (B) Phosphorylation of Y419 without dephosphorylation of Y530 of c-Src by E2 in ECs. Pretreated cells were treated with 30 nM E2 for the indicated time, followed by lysate immunoblot with pY419-Src, pY530-Src, npY530 (non-phospho-Y530)-Src, and c-Src antibodies. PDGF (5 ng/ml) treatment demonstrates a typical, parallel Y419 phosphorylation and Y530 dephosphorylation response using the same antibodies.

estrogen-triggered signals. E2 stimulation of aortic rings was also performed in the presence of prostaglandin synthesis inhibition (indomethacin). Although the resting tone was greater in the presence of indomethacin, the vasodilating response to E2 was unaffected (data not shown). L-NAME pretreatment followed by PE challenge was also used to evaluate basal NO release. PE-induced, basal aortic tone in c-Src^{-/-} rings was equal to that observed in L-NAME-treated wild-type rings (data not shown). These data implicate c-Src as a physiologically relevant modulator for basal and E2-induced vasorelaxation in murine aortas.

E2-Induced c-Src Phosphorylation Status and c-Src Requirement in eNOS Activation.

To document a c-Src requirement in E2-induced eNOS activation, we compared responsiveness in murine embryonic fibroblasts derived from embryos lacking all three major Src kinase members, Yes, Fyn, and c-Src (SYF^{-/-}), to those observed in the same cells with c-Src reconstitution by stable c-Src expression (YF^{-/-}). In triple knockout SYF^{-/-} murine embryonic fibroblasts that heterologously express human ER46 and bovine eNOS, E2 failed to activate eNOS. However, E2 stimulation did increase eNOS activity (2.7 ± 0.4 -fold) in YF^{-/-}, Src^{+/+} cells (Fig. 2A). Expressed ER46 and eNOS levels in these transfected cells were equal (SI Fig. 8). Phosphorylation of c-Src Y419 (avian Y416) with concomitant Y530 (avian Y527) dephosphorylation is a consequence of c-Src activation and often leads to full kinase activity (4). In human umbilical vein ECs that contain both full-length ER α (referred to as ER α , unless otherwise indicated) and ER46, and EA.hy926 cells (an immortalized human umbilical vein EC line) that conditionally express ER46 only (2), E2 stimulated phosphorylation of c-Src on Y419 residue in 5–15 min while the levels of phospho-Y530 and non-phospho-Y530 remained unchanged (Fig. 2B). This is in contrast to PDGF-induced, parallel EC c-Src phosphorylation and dephosphorylation. Thus, the E2-induced eNOS activation

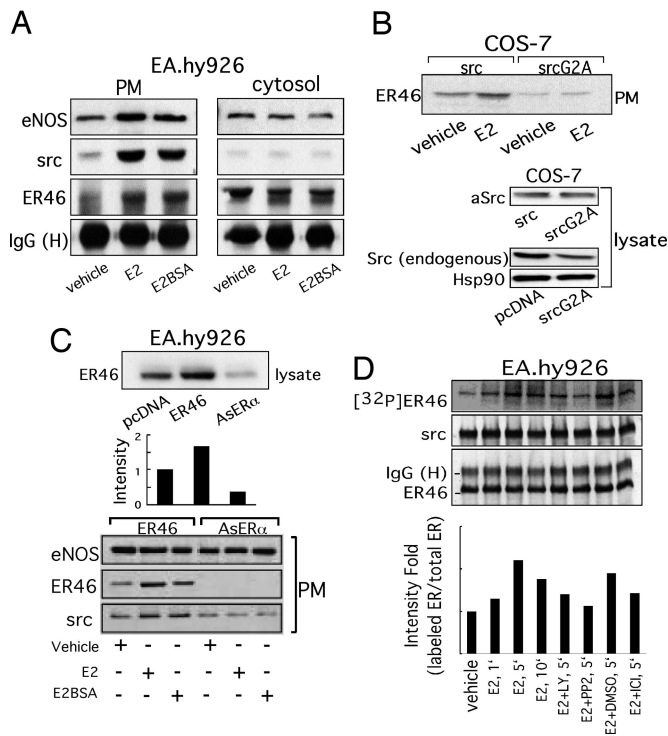


Fig. 3. Assembly of a functional ER46–Src–eNOS macrocomplex in plasma membranes. (A) Complex formation in EC plasma membranes. Isolated plasma membranes and cytosol from E2-treated or E2BSA-treated (30 nM, 10 min) EA.hy926 cells were used for coimmunoprecipitation with ER antibody (F-10), followed by immunoblot with c-Src and eNOS antibodies. (B and C) Interdependent incorporation of c-Src and ER46 in plasma membranes. Electroporated EA.hy926 and Lipofectamine-transfected COS-7 cells were treated with 30 nM E2 or E2BSA for 10 min, fractionated, and immunoblotted. (D) Phosphorylation of ER46 by c-Src *in vitro*. ER46-centered complexes were immunoprecipitated with ER antibody (F-10) from EA.hy926 cell lysate and treated with 30 nM E2 and indicated inhibitors (10 μ M LY 294002, 10 μ M ICI 182,780, and 10 μ M PP2) in the presence of [γ - 32 P]ATP *in vitro*, followed by protein fractionation, autoradiography, and immunoblot.

requires the presence of c-Src, which becomes phosphorylated on Y419 in the absence of c-Src Y530 dephosphorylation.

ER46–c-Src Interdependence for Plasma Membrane Localization. Several features of ER, c-Src, and eNOS membrane targeting have been described (3, 11, 12), but molecular interdependence of such has not been addressed. c-Src was coimmunoprecipitated with ER46 and eNOS from EA.hy926 plasma membranes but not from the cytosol. E2 or membrane-impermeant E2BSA enhanced the association of ER46, c-Src, and eNOS in the membrane (Fig. 3A). Decrease of ER46 expression (65%) with antisense ER α in EA.hy926 cells (Fig. 3C) or overexpression of myristoylation-deficient c-Src mutants in ER46-transfected COS-7 cells diminished the plasma membrane appearance of c-Src and ER46 (Fig. 3B and C). The levels of eNOS in plasma membranes (Fig. 3C) and of total Hsp90 (Fig. 3B) in cell lysates were not altered by AsER α and SrcG2A expression, indicating the specificity of changes in targeted genes. Of note, expression of exogenous avian c-Src (aSrc) in COS-7 cells reduced the levels of endogenous c-Src. This may explain why heterologous c-Src expression apparently masked the role of endogenous c-Src in these studies (Figs. 3 and 4). These results indicate that, in addition to palmitoylation and myristoylation, known to be important for membrane localization of ER46 (2) and c-Src (5), respectively, ER46 and c-Src are interdependent for membrane incorporation, possibly because of their protein–protein interaction.

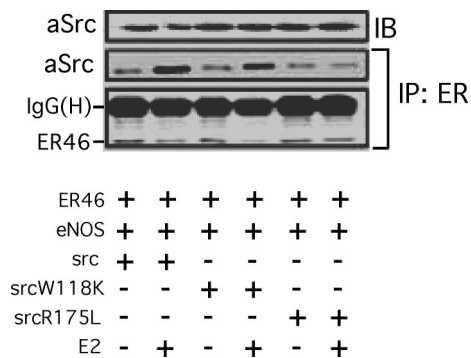


Fig. 4. Coimmunoprecipitation of ER with c-Src variants. COS-7 cells were transfected with ER46, eNOS, and the indicated avian c-Srcs, E2-deprived for 48 h, serum-starved for 12 h, and stimulated with 33 nM E2 for 10 min. Cell lysates were used for immunoprecipitation with F-10 antibody, and the coimmunoprecipitated avian c-Src was examined by immunoblotting. SrcW118K, c-Src SH3 mutant; SrcR175L, c-Src SH2 mutant.

ER46–c-Src Interaction Domains. To define ER46 and c-Src domains that confer the aforementioned, critical interactions, a series of avian (to distinguish between endogenous and transfected) c-Src mutants were cotransfected with ER46 (and, in activation experiments, with eNOS) into COS-7 cells, followed by coimmunoprecipitations. Before doing so, metabolic labeling experiments were performed to determine whether E2 induces a c-Src-dependent phosphorylation of ER46 in EC. In EA.hy926 cell ER46–Src–eNOS immunocomplexes (Fig. 3D), E2 stimulates ER46 phosphorylation *in vitro*, a process blocked by both the Src inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2) (95%) and the pure ER antagonist ICI 182,780 (70%). Because Y537 is the only known c-Src substrate residue in ER α (9, 13), it is likely that this phosphorylation event enhances, or is even responsible for, the ER46–c-Src interaction in EC. This is supported by our finding that, after E2 stimulation, ER46 antibody coimmunoprecipitated wild-type aSrc and the SH3 mutant aSrcW118L, but not the SH2 mutant aSrcR175L (Fig. 4), one shown to abrogate c-Src interactions with phosphotyrosine-containing proteins (14). As noted above, c-Src is often self-restrained to an inactive conformation by both intramolecular and intermolecular interactions between its SH2 domain and phospho-Y530 and between its SH3 domain and SH2-kinase linker domains. To determine whether the c-Src substrate-accessible open structure and/or kinase activation are required for c-Src localization in membrane ER46 complexes, the avian c-Src constructs including wild-type Src, SrcY527F (open and active), SrcK295M (kinase-dead), and SrcY527F/K295M (substrate-accessible but kinase-dead) were separately expressed in ER46-reconstituted COS-7 cells (Fig. 5A). Wild-type Src expression supported E2BSA-stimulated membrane ER46 recruitment. Conversely, SrcK295M, a kinase-dead mutant that is likely to adapt an inactive conformation, radically abolished (95%) membrane localization of ER46 in the presence and absence of E2BSA challenge. Both SrcY527F and SrcY527F/K295M elevated the basal level of membrane ER46. In these two transfectants, E2BSA-induced ER46 recruitment was not observed, likely because membrane ER46 levels were already near maximal. Furthermore, the changes of membrane ER46 expression conferred by wild-type Src or Src mutants correlate with the ability of ER46 to bind membrane-impermeant ligands, determined by HRP activity associated with membrane ER46 after E2HRP (membrane-impermeant) treatment of reconstituted COS-7 (Fig. 5B), SYF $^{-/-}$ (Fig. 5C), and YF $^{-/-}$, Src $^{+/+}$ (Fig. 5D) cells in culture. SrcY527F and SrcY527F/K295M increased (2.4 \pm 0.2-fold and 2.6 \pm 0.1-fold,

eNOS activation, have been observed (21). Thyroid hormone can trigger this rapid activation response through engagement of the predominant thyroid hormone receptor in EC, TR α_1 . Thus, there are clearly multiple potential rapid signaling pathways activated by ER engagement and redundancy of receptor classes that, upon engagement, can facilitate identical outcomes of endothelial activation, namely NO release.

Our studies indicate that c-Src is required for E2-induced vasorelaxation. Based on the inhibition of vasodilation observed when aortic rings were pretreated with L-NAME or a conventional ER antagonist, the induced vascular responses are, at least in part, NO- and ER-dependent. These *ex vivo* data are entirely consistent with our (and others') prior *in vitro* findings that E2 triggers an ER-dependent, c-Src/phosphatidylinositol 3-kinase/Akt-mediated activation of eNOS, with consequent EC NO release (2, 3, 12). Recently, E2 has been shown to induce Akt S473 phosphorylation and rapid NO production in isolated cerebral blood vessels (22), also consistent with both our *ex vivo* and *in vitro* data. In this work, in addition to demonstrating the vascular tissue correlate of our *in vitro* data, our efforts were directed at defining molecular interaction domains between ER and c-Src and assessing the importance of classical c-Src structure and function and the interdependence of these two molecules for plasma membrane targeting, all in the context of eNOS activation. Indeed, the molecular determinants of these activation events include an interdependent plasma membrane targeting with a subset of c-Src bearing a substrate-accessible conformation, as well as a function-modifiable Src kinase domain.

The c-Src C-terminal Y530 (avian 527) is an important regulatory phosphorylation site. Under basal conditions, 90–95% of the c-Src molecules are phosphorylated by constitutively active tyrosine kinases such as Csk and Chk (23). The p-Y530 binds intramolecularly to its SH2 domain, generally believed to prevent substrate accessibility and the required Y419 (avian 416) phosphorylation. A key activation switch often occurs when phosphatases such as PTP α dephosphorylate p-Y530 with consequent dissociation from the SH2 binding pocket. In our study, c-Src presents a substrate-accessible conformation without Y530 dephosphorylation (Fig. 2). Instead, protein–protein interactions, perhaps disrupting the intramolecular rigidity without affecting Y530 dephosphorylation (24, 25), may largely contribute to opening c-Src. The availability of a low level of c-Src kinase activity in resting cells (13) and the increasing association of ER46 with E2 stimulation (Fig. 3A) may favor homotypic (26, 27) and heterotypic (24, 25) protein interactions with c-Src and further enforce c-Src “opening.” Indeed, it has been demonstrated that doubly phosphorylated c-Src can be active, such that Y419 phosphorylation can override the p-Y530-directed inhibition (28). The balance of tyrosine kinase (Csk and Chk) and phosphatase (PTP α) activity, in both basal and stimulated states, is partially responsible for the state of Src activation. We are currently investigating this balance in resting and E2-stimulated EC.

A number of posttranslational modifications and protein–protein interactions have been shown to direct ER localization to plasma membranes. These include palmitoylation on C447 (29) and caveolin-1 interaction with ER α S522 (30), the latter promoting targeting to specialized membrane rafts, caveolae, in EC. Furthermore, multiple proteins, including the modulator of nongenomic activity of ER (MNAR) and striatin, serve as scaffold molecules for membrane complexes containing ER with c-Src (31) and Gai (32), respectively. Here we demonstrate that productive membrane targeting of c-Src (abrogated by the SrcG2A myristoylation mutant) is also important for maximal plasma membrane targeting of ER. We observed a rapid, E2-stimulated, Src- and ER-dependent ER46 phosphorylation. Although we do not map this phospho-site, others have demonstrated that ER α Y537, when inducibly phosphorylated, binds to the c-Src SH2 domain (31). Because the SrcR175L SH2 mutant loses its E2-induced association with ER46 (Fig. 4), it is likely that this Y537 is the critically stimulated phosphorylation/

activation site, responsible for triggering rapid signaling cascades, including those leading to eNOS activation and EC NO release.

We have used EC that, in our experimental conditions, express the truncated ER α splice form ER46 (2). We have also used recombinant ER46 expression in heterologous systems to dissect these molecular interactions *in vitro*. We find that all of the membrane targeting, molecular complex assembly, and rapid activation responses are achievable with ER46 in EC. It is clear that full-length ER α can also localize to endothelial caveolar structures (31), and the predominance of a given isoform in a physiologically or pathologically relevant vascular context is not known. Both full-length and truncated (\approx 50 kDa) ERs have been coimmunoprecipitated with eNOS from rat cerebral vessels (22). Also, the original ER α gene-deleted mouse (ER α KO_{Chapel Hill}), targeted for deletion within exon 1, was reexamined after vascular responses were found to be maintained. Indeed, truncation variants resulting from both splice and internal translation initiation sites within exon 2, ER55 and ER46, were demonstrated in the vasculature of that mouse and were capable of mediating estrogen responses (24, 33, 34).

Our findings are consistent with human vascular endothelial expression of ER46, a receptor isoform that efficiently promotes the aforementioned, multimeric signalsome and, upon engagement by ligand, facilitates the signal transduction cascade, resulting in NO release (2, 3). Given the major efforts directed at evaluating ovarian steroid replacement effects on the cardiovascular status of postmenopausal women, careful dissection of rapid, membrane-initiated, ER-dependent signaling is timely and critical to more carefully targeted future therapies.

Materials and Methods

Materials and Reagents. The anti-ER α antibody (F-10), all secondary antibodies, and protein A/G-plus reagent were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibodies were used for c-Src (Ab-1; Oncogene Research Products, La Jolla, CA), avian Src (EC-10; Upstate Biotechnology, Lake Placid, NY), and eNOS (BD Transduction Laboratories, San Jose, CA), whereas rabbit polyclonal antibodies were used for phospho-S1177-eNOS, phospho-Y416-Src, phospho-Y527-Src, and nonphospho-Y527-Src from Cell Signaling Technology (Beverly, MA). E2, water-soluble E2, PE hydrochloride, acetylcholine chloride, sodium nitroprusside, and L-NAME were purchased from Sigma (St. Louis, MO). LY294002 and PP2 were purchased from Calbiochem (La Jolla, CA), and ICI 182,780 was from Zeneca Pharmaceuticals (Wilmington, DE). All other reagents were purchased from Sigma unless otherwise noted. All cell culture media were purchased from GIBCO/BRL (Gaithersburg, MD).

Plasmids. ER46 (amino acids 174–595 of ER α) was obtained by PCR amplification with primers containing a BamHI site using hER19Pu as a template. hER19Pu was gift from P. Chambon (Université Louis Pasteur, Strasbourg, France). The amplified fragment was then subcloned into pSG5 or pCMV-Tag3c (Stratagene). Insert for antisense ER α (AsER α) construct was digested by BamHI from hER0Pu, containing human ER α , and then subcloned into pCMV-Tag3c. The numbering of ER α refers to human ER α (Swiss-Prot accession no. P03372). The plasmids containing avian Src mutants such as SrcG2A, SrcW118K, and SrcR175L were prepared with the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) using avian Src/CA10 as a template. Avian Src constructs containing SrcY527F, SrcK295M, and SrcY527F/K295M were obtained from P. Schwartzberg (National Institutes of Health, Bethesda, MD) and J. Brugge (Harvard University, Cambridge, MA). The numbering of avian Src mutants and human Src refers to the sequences of chicken Src (Swiss-Prot accession no. P00523) and human Src (Swiss-Prot accession no. P12931), respectively. Constructs were all verified by sequencing.

Cell Culture and Transfection. Murine embryonic fibroblasts derived from embryos deficient in c-Src, Yes, and Fyn (SYF^{-/-}) or fibroblasts derived from embryos lacking both Yes and Fyn but maintaining normal levels of c-Src (YF^{-/-}Src^{+/+}) were obtained from American Type Culture Collection (Manassas, VA). Cells were subcultured and maintained as described previously (3, 11). The immortalized vascular EC line EA.hy926 and human umbilical vein ECs were maintained as described previously (3). For pretreatment, cells were E2-deprived for 48 h in phenol-red-free DMEM or M199 containing 15% gelding horse serum and serum-starved for 12 h in phenol-red-free DMEM or M199 supplemented with 0.25% BSA. Cells were transfected by electroporation. Alternatively, Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used according to the manufacturer's guidelines.

Subcellular Fractionation. Plasma membranes were isolated by a cationic colloidal silica isolation technique (35). Briefly, cells were dissociated in dissociation buffer (GIBCO/BRL), resuspended in 1% cationic colloidal silica in plasma membrane coating buffer [PMCB; 20 mM Mes, pH 5.5/150 mM NaCl/250 mM sorbitol/1 mM Na₃VO₄/1 mM NaF/protease inhibitor mixture (Roche, Indianapolis, IN)], overcoated in 1 mg/ml polyacrylate in PMCB, mechanically lysed in hypotonic buffer (2.5 mM imidazole, pH 7.0/5 mM EDTA) by a probe sonicator (Fisher, Pittsburgh, PA), mixed with an equal volume of 100% Nycodenz, layered onto 70% Nycodenz, topped with hypotonic buffer, and sedimented at 60,000 × g for 40 min at 4°C. Soluble proteins and plasma membranes were separately collected, dialyzed against PBS for 2 h at 4°C, and recovered in PBS containing 1 mM Na₃VO₄.

ER46 Phosphorylation by E2 in Vitro. Cells were disrupted for 4 h at 4°C in lysis buffer (20 mM Tris-HCl, pH 7.4/1% Nonidet P-40/137 mM NaCl/1 mM CaCl₂/1 mM MgCl₂/0.1 mM Na₃VO₄). Aliquots of the supernatant containing 200 μg of protein were precleared by 0.4 μg of irrelevant IgG (OKT3) and protein A/G-plus reagent for 1 h at 4°C and were subjected to immunoprecipitation with anti-ERα antibody (F-10) in the presence of protease inhibitor mixture (Roche) overnight at 4°C. Immunocomplexes were then extensively washed in lysis buffer three times and once in wash buffer (10 mM Tris-HCl, pH 7.5/0.3 M sucrose/1% Nonidet P-40/10% glycerol/0.1

mM EDTA/0.1 mM EGTA/1 mM DTT/1 mM Na₃VO₄), after which the immunocomplexes were resuspended in 20 μl of kinase assay buffer [20 mM Tris-HCl, pH 7.5/5 mM MgCl₂/0.2 mM CaCl₂/0.5 mM DTT/10 μCi of [³²P]ATP (>4,000 Ci/mmol; 5 μM ATP)], with and without 30 nM E2 and inhibitors as described in indicated figure legends. Incubation was maintained at room temperature for 15 min and terminated by the addition of SDS/PAGE sample buffer.

eNOS Activity Assay. eNOS activity was monitored by L-[³H]citrulline production from L-[³H]arginine as described previously (36). Briefly, protein samples were incubated in reaction buffer [1 mM L-arginine/100 mM NADPH/1 mM tetrahydrobiopterin/0.2 μCi of L-[³H]arginine (>66 Ci/mmol) per reaction] for 15 min at 37°C, separated by Dowex-50W ion-exchange chromatography in 20 mM Hepes (pH 5.5), 2 mM EDTA, and 2 mM EGTA, and the flow-through was used for liquid scintillation counting.

Surface E2-HRP Binding. Cell monolayer was incubated with 600 nM E2-HRP for 10 min on ice in the presence and absence of 30 μM E2BSA, washed in PBS, homogenized in lysis buffer [20 mM Tris-HCl, pH 7.4/100 mM NaCl/1% Nonidet P-40/1 mM CaCl₂/1 mM MgCl₂/1 mM Na₃VO₄/protease inhibitor mixture (Roche)], immunoprecipitated by F10 or OKT3 IgG (0.2 μg/mg of protein), incubated in 50 μl of substrate reagent (BD Pharmingen) at room temperature until color developed, terminated with 50 μl of 1 M H₃PO₄, and monitored at 459 nm by spectrophotometry.

Ex Vivo Vasomotion. Vasodilation experiments were performed as described previously (37) using anatomically dissected thoracic aorta rings from PBS-perfused control and Src^{-/-} mice. Control thoracic aorta rings were from either C57BL/6J or Src^{+/+} littermates, which yielded similar results.

We thank Dana Brenckle for assistance with the manuscript and all those generously providing key plasmids. This work was supported by National Institutes of Health Grants R01HL 67182 (to J.R.B.) and R01AR042927 (to R.B.) and by a grant from the Raymond and Beverly Sackler Foundation (to J.R.B.).

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