Src Kinase Mediates Phosphatidylinositol 3-Kinase/Akt-dependent Rapid Endothelial Nitric-oxide Synthase Activation by Estrogen*

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17β-Estradiol activates endothelial nitric oxide synthase (eNOS), enhancing nitric oxide (NO) release from endothelial cells via the phosphatidylinositol 3-kinase (PI3-kinase)/Akt pathway. The upstream regulators of this pathway are unknown. We now demonstrate that 17β-estradiol rapidly activates eNOS through Src kinase in human endothelial cells. The Src family kinase specific-inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) abrogates 17β-estradiol-induced Akt phosphorylation but did not inhibit NO release from cells transduced with a constitutively active Akt. PP2 abrogated 17β-estradiol-induced activation of PI3-kinase, indicating that the PP2-inhibitable kinase is upstream of PI3-kinase and Akt. A 17β-estradiol-induced estrogen receptor/c-Src association correlated with rapid c-Src phosphorylation. Moreover, transfection of kinase-dead c-Src inhibited 17β-estradiol-induced Akt phosphorylation, whereas constitutively active c-Src increased basal Akt phosphorylation. Estrogen stimulation of murine embryonic fibroblasts with homozygous deletions of the c-src, fn, and yes genes failed to induce Akt phosphorylation, whereas cells maintaining c-Src expression demonstrated estrogen-induced Akt activation. Estrogen rapidly activated c-Src inducing an estrogen receptor, c-Src, and P85 (regulatory subunit of PI3-kinase) complex formation. This complex formation results in the successive activation of PI3-kinase, Akt, and eNOS with consequent enhanced NO release, implicating c-Src as a critical upstream regulator of the estrogen-stimulated PI3-kinase/Akt/eNOS pathway.

The cardioprotective effects of estrogen are diverse, including both rapid non-genomic and delayed genomic effects on the blood vessel wall (reviewed in Ref. 1). Specific, rapid genomic effects, such as moderation of vasoconstrictor tone, have been linked to an estrogen-stimulated increase in bioavailable nitric oxide (NO) (2–4). 17β-estradiol (E2) treatment of human endothelial cells (EC) induces rapid release of NO by estrogen receptor (ER)-dependent activation of endothelial nitric oxide synthase (eNOS) (5). Many factors regulate eNOS enzyme activity, including fatty acid modification, subcellular localization, and binding to numerous proteins and cofactors, including calmodulin, caveolin-1, the 90-kDa heat shock protein (HSP90), and tetrahydrobiopterin (see Ref. 6 for review). eNOS is a Ca2+/calmodulin-dependent enzyme, the activity of which is also regulated by phosphorylation. Specific phosphorylation of eNOS by the serine/threonine kinase Akt renders the enzyme more active at much lower Ca2+ concentrations (7, 8). We demonstrated previously that the ER-dependent activation of eNOS occurs at resting Ca2+ concentrations and requires activation of the phosphatidylinositol-3-OH kinase (PI3-kinase)/Akt pathway (9). The regulatory subunit of PI3-kinase, P85, acts to stabilize and inhibit the catalytic activity of PI3-kinase. Recently, ER was shown to specifically bind to P85 in vitro (10). The E2-induced association correlated with increases in PI3-kinase activity in EC. However, the specific mechanism for E2 activation of PI3-kinase is not known.

Evidence is emerging that membrane forms of steroid hormone receptors exist and participate in signaling pathways (11–14). The activity of E2 at the cell membrane has been shown in EC, neurons, and breast cancer cell lines. We previously determined that rapid E2 activation of eNOS and MAP kinase occurs through a membrane-associated ER (9, 12). The EC line EAhY.926 used in these experiments exhibits rapid E2-induced signaling but is unable to stimulate ER-dependent gene transactivation. Additionally, EAhY.926 cells do not express the traditional 66-kDa ERα or ERβ but express a 46-kDa protein immunoreactive with C-terminal ER antibodies. Recently, a protein of similar size reactive with E2 and anti-ER antibodies was found to be associated with the plasma membrane in MCF-7 cells (13, 14). Additionally, a 46-kDa putative ER, reactive with anti-ER antibodies, was found in wild-type and in the initial ERα knockout mouse. This form of the receptor was thought to be responsible for E2 enhancement of basal NO production in the initial ERα knockout mice, because this E2 effect was lost in the complete ERα knockout mouse (15). In human ECs expressing both the 66- and the 46-kDa receptor, both rapid signaling to MAP kinase and gene transactivation of estrogen-responsive element-luciferase reporter was stimulated with E2 treatment (12). As previously indicated, the specific mechanism of membrane-associated ER coupling to activated protein; DMEM, Dulbecco’s modified Eagle’s medium; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; β-gal, β-galactosidase; myr-Akt, membrane-targeted, myristoylated Akt; HUVEC, human umbilical vein endothelial cell; PIP, phosphatidylinositol phosphate.
c-Src Mediates Estrogen Receptor-dependent eNOS Activation

P85 is unknown. E2-mediated actions are sensitive to serine/threonine and tyrosine kinase inhibition. Previously, the activation of the tyrosine kinase c-Src was associated with rapid E2 effects in breast cancer cells (16, 17). Src activation induces MAP kinase through a Src/Grb2/Ras signaling cascade. In addition to MAP kinase, Ras-GTP has been shown to be bound and activate PI3-kinase. Because E2 rapidly activates both EC MAP kinase and PI3-kinase, we investigated the ability of E2 to activate Src kinase in human EC and whether the consequence of this activation included activation of PI3-kinase, Akt, eNOS, and MAP kinase. Here, we present evidence that the non-receptor tyrosine kinase, c-Src, is rapidly activated in EC upon stimulation by E2. This activation leads to formation of a functional signaling complex composed of ER, c-Src, and P85.

EXPERIMENTAL PROCEDURES

Materials—E2 and ionomycin were purchased from Sigma. Stock solutions were prepared in ethanol with final ethanol concentrations less than 0.1%. Stock solutions of LY294002 (Calbiochem) and ICI 182,780 (Zeneca Pharmaceuticals) were prepared in Me2SO, with final Me2SO concentrations less than 0.1%. Anti-phosphorylated Akt, anti-Akt, anti-phospho-p60, and anti-phosphorylated eNOS were purchased from Cell Signaling. Anti-P85 and anti-c-Src were purchased from Santa Cruz. Anti-eNOS antibody was purchased from BD Transduction Laboratories. All other reagents were purchased from Sigma unless otherwise noted.

Cell Culture—The EC line EAhy.926, described previously (9, 18), was maintained in DMEM and 10% fetal bovine serum, supplemented with 5 mM hypoxanthine, 0.8 mM thymidine, and 20 mM aminopterin. Human umbilical vein EC (HUVEC) were isolated and maintained as described previously (5). Murine embryonic fibroblasts derived from human umbilical vein EC (HUVEC) were isolated and maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum and 100 U/ml penicillin and 100 μg/ml streptomycin.

The anti-ERα, described previously (8), were obtained from K. Walsh (St. Elizabeth’s Medical Center, Boston, MA). Monolayers were incubated with the recombinant adeno-viruses at a multiplicity of infection of 100 for β-gal and myr-Akt. After infection, E2-free medium was added for the cell recovery period followed by serum starvation in phenol red-free DMEM plus 0.1% bovine serum albumin. Adeno-virally infected cells were stimulated as described in the figure legends.

Transient Transfection—Cell monolayers were incubated with empty vector (pcDNA3), kinase dead Src kinase (Src K295M), or constitutively active Src kinase (Src Y527F) and pEGFP (Roche Molecular Biochemicals) at a 1:6 DNA-to-lipid ratio according to the manufacturer’s directions in E2-free medium. After transfection, cells were serum-starved in phenol red-free DMEM and 1% bovine serum albumin before stimulation with E2.

RESULTS

Effect of Src Family Kinase Inhibition on NO Release—We demonstrated previously that induced NO release occurs through activation of E2-stimulated PI3-kinase and Akt activation, resulting in phosphorylation and enhanced activation of eNOS (9). Once Akt is targeted to the membrane via a PI3-kinase-dependent mechanism, it can be phosphorylated on at least two residues, Ser473 and Thr308. Phosphorylation of Thr308 is thought to be largely constitutive, whereas Ser473 phosphorylation is highly inducible. Thus “activation” of Akt is almost exclusively measured by Ser473 phosphorylation (20). Recently, in addition to phosphorylation of Ser473 and Thr308, Akt has been shown to be phosphorylated on Tyr1235 and Tyr1236 by Src kinase (21). This tyrosine phosphorylation is thought to be important for full activation of Akt but is independent of phosphoinositide phosphorylation. Phosphorylation of Akt on Ser473 and Thr308 is a required step in Akt-dependent eNOS activation. This phosphorylation further activates eNOS, a required step in Akt-dependent eNOS activation. Therefore, we attempted to determine where in the pathway Src kinase was a primary upstream mediator of the signal transduction pathway leading to E2-mediated NO release. EC were pretreated with PP2, a pharmacological inhibitor specific for Src family tyrosine kinases (22, 23), or treated with vehicle control before stimulation. Cells were infected with recombinant adenovirus encoding either myr-Akt or kinase dead Src kinase (Src Y527F). Therefore, we attempted to determine whether Src kinase was a primary upstream mediator of the signal transduction pathway leading to E2-mediated NO release.

Effect of PP2 on Constitutively Active Akt-Enhanced NO Release—To begin dissecting the level at which Src kinase transduces the aforementioned E2-stimulated responses, EAhy.926 cells were infected with recombinant adeno-virus encoding either β-gal as a control or a membrane targeted and thus constitutively active myr-Akt. After infection, the cells were pretreated with PP2, followed by E2 or vehicle stimulation for 30 min, and the NO release was quantified. PP2 has no effect on NO release induced by constitutively active Akt (Fig. 2), indicating that Akt itself is not a critical substrate for Src kinase. That is, Src kinase-mediated Akt tyrosine phosphorylation is not a required step in Akt-dependent eNOS activation. This demonstrates that a Src kinase is playing a role upstream of Akt-mediated eNOS phosphorylation. Because PP2 was unable to prevent NO release induced by constitutively active Akt, it was important to determine where in the pathway Src kinase was involved. PP2 abrogates E2-induced phosphorylation of Akt on Ser473 in immortalized EAhy.926 EC (Fig. 3A) and in...
HUEVEC (Fig. 3B), indicating Src kinase involvement in the primary activation of Akt (Fig. 3). Phosphorylation of a downstream target of E2-activated Akt, eNOS Ser1177, was inhibited by pretreatment of HUEVEC with PP2 (Fig. 3C), correlating with PP2 inhibition of E2-stimulated NO release (Fig. 1). These data also indicate that the E2-induced signaling responses seen in the immortalized EAhy.926 cells are functionally identical to that seen in primary HUEVEC.

Effect of E2 Stimulation on EC c-Src—Although the PP2 data do not define the precise Src kinase involved, c-Src is rapidly phosphorylated in response to E2 in mammary tumor cell lines and osteoblasts (17, 24–26). In these cells, Src kinase activation results in the induction of the Src/Ras/Erk signal transduction pathway. We have previously demonstrated rapid EC Erk1/2 activation in response to E2 (12). Human EC, including EAhy.926 cells, contain easily detectable levels of c-Src. Therefore, E2-induced c-Src activation was evaluated. c-Src activation kinase requires carboxy-terminal Tyr416 dephosphorylation and subsequent kinase domain Tyr416 autophosphorylation (27). Fig. 4A demonstrates induced c-Src Tyr416 phosphorylation within 2 min of E2 stimulation. As with all other E2-stimulated rapid signaling responses we have observed in human EC (5, 9, 12, 28), this activation is completely inhibited by the conventional ER antagonist ICI 182,780, indicating that this is an ER-mediated event (see below). PP2 also abrogates E2-induced c-Src phosphorylation, consistent with the requirement for autophosphorylation. However, E2-induced c-Src phosphorylation was not inhibited by the specific PI3-kinase inhibitor LY294002, indicating E2 activation of Src kinase occurs before activation of the PI3-kinase/Akt pathway.

Although c-Src phosphorylation/activation is clearly E2-induced in EC (Fig. 4A) and in osteoclasts (16, 25, 29), the precise mechanism has not been defined. In osteoclasts, an interaction between ligand-activated steroid receptors and c-Src seems required for kinase activity (16, 25, 29). Western blots performed on concompontoprecipitates from E2-stimulated EAhy.926 cells demonstrated a rapidly induced c-Src/ER association (Fig. 4B). The anti-ER antibody used is a monoclonal antibody directed at the carboxyl terminus of ERα. In human EC, including EAhy.926, the antibody blots/immunoprecipitates a 46-kDa protein that we believe to be the predominant membrane-associated ER in these cells (Ref. 12, and below). Thus, E2 stimulation promotes the formation of a putative signaling complex between the 46-kDa, signal-transducing ER and c-Src. As might be expected from our previous results, PP2 also inhibited E2- but not ionomycin-stimulated Erk1/2 activation (data not shown). These data support the idea that E2-induced c-Src activation results in parallel activation of the MAP kinase (Erk1/2) and Akt pathways, the latter of which results in eNOS phosphorylation and augmented NO release.

The Role of Src Kinase in E2-induced PI3-kinase Activation—We have previously demonstrated that E2-activated NO
release can be completely inhibited by the PI3-kinase inhibitor LY294002 (9), indicating an absolute requirement for PI3-kinase. It was recently demonstrated that ERα can associate with the regulatory subunit of PI3-kinase, P85, and that this association correlates with increased production of phosphatidylinositol 3,4,5-trisphosphate (PIP) (10). The mechanism by which ligand-induced ER/P85 association activates PI3-kinase remains to be determined. In EAhy.926 cells, which do not express the full-length ER, we thus evaluated whether this association correlated with increased production of phosphatidylinositol 3-phosphate (PIP), with maximum levels achieved in 10 min. These increases in PIP were completely abrogated by ER antagonist ICI 182,780, LY294002 (LY, 10 μM), or PP2 (10 μM) for 30 min before activation. E2 was added to the monolayer in serum-free medium for the indicated times. A, cells were washed, lysed and immunoblotted with phosphorylation-specific c-Src antibody (pSrc). Membranes were reprobed with total c-Src antibody (c-Src). B, E2 (10 ng/ml) was added to the monolayer in serum-free medium for the indicated times. Cells were washed, lysed, and immunoprecipitated with anti-c-Src antibody-agarose conjugate. Immunoprecipitates (IP) were immunoblotted with anti-ER antibody and reprobed with anti-c-Src antibody. The ratio of ER that was co-immunoprecipitated with c-Src was determined by densitometry based upon total c-Src detected in the immunoblots. WB, Western blot.

**Fig. 5. Effect of E2 on ER/P85/c-Src interaction.** A, E2-deprived and serum-starved EC monolayers were stimulated with E2 (10 ng/ml) for 15 min. Cells were washed, lysed, and immunoprecipitated with anti-P85 antibody. Immunoprecipitates (IP) were immunoblotted with anti-P85 antibody and reprobed with anti-ER antibody. WB, Western blot. B and C, E2-deprived and serum-starved EC monolayers were pretreated with ICI 182,780 (ICI, 10 μM), PP2 (10 μM), or vehicle for 30 min and then stimulated with E2 for 10 min. Cells were washed, lysed, and immunoprecipitated with anti-ER or anti-c-Src antibodies. Immunoprecipitates were immunoblotted with anti-P85 antibody. Membranes were reprobed with anti-ER antibody or anti-c-Src antibody.
stimulated NO release in the absence of a Ca$^{2+}$ flux. However, the mechanism of E2-induced Akt phosphorylation remains unknown.

Our data, and those of others, have defined PI3-kinase as a critical upstream activator in the E2-stimulated Akt/eNOS activation pathway (9, 10). In fact, a direct interaction between ER and P85, the regulatory subunit of PI3-kinase, has been demonstrated, correlating with increased PI3-kinase activity (10). However, the E2-stimulated molecular switches responsible for the ER/PI3-kinase association are not defined. There are several reasons to suspect that Src family kinases could be the link between ER and PI3-kinase. First, we and others have demonstrated, in parallel to Akt activation, that E2 stimulation of EC results in rapid ERK$\_12$ activation (12, 30). This response resembles that mediated by receptor tyrosine kinases, which, in some cases, recruit Src family kinases as a part of a MAP kinase cascade. Second, P85 has been shown to be a Src kinase (lck and abl) substrate (33–35), and fyn, lyn, and lck can, through their SH3 domains, interact with P85 (36–41). Third, estrogen-induced c-Src phosphorylation has been demonstrated in osteoclasts and breast cancer cell lines (24, 29).

Here, we provide the first demonstration of ER-dependent c-Src activation in EC, and that this activation provides a functional link between ER engagement and the PI3-kinase/ Akt/eNOS pathway. A pharmacological inhibitor of the Src family tyrosine kinases inhibited not only Akt activation and NO release but also PI3-kinase dependent generation of phosphatidylinositol phosphates, indicating that Src activation is upstream of PI3-kinase. The c-Src specificity was documented by inhibiting E2-induced Akt phosphorylation with a kinase-dead c-Src. We now demonstrate an estrogen-stimulated molecular complex formation, between ER, P85, and c-Src, that includes activated c-Src, phosphorylated within 2 min of E2 treatment. The basis and direct consequence of a P85/c-Src interaction remain to be determined, although several possibilities exist. As noted above, P85 was shown to be specifically phosphorylated on Tyr$^{537}$ by the Src kinases lck and abl (33–35), and PI3-kinase has been shown to be a preferential substrate for c-Src (42, 43). It is also possible that estrogen-activated c-Src could tyrosine phosphorylate docking proteins containing binding sites for the SH2 domain of P85, thus alleviating, upon interaction, the inhibitory constraint on the PI3-kinase P110 catalytic subunit (44, 45). Alternatively, the SH3 domains of several Src kinases have been shown to bind directly to P85 and regulate its activity (36–41). This includes c-Src that, in osteoclasts, interacts directly through its SH3 domain with P85, in response to colony stimulating factor-1 (46).

Although we believe that these rapid signaling responses to estrogen have important implications in vascular tissue, other ligand-activated steroid hormone receptors may have similar effects. Engagement of the androgen receptor, but not the progesterone receptor, can result in phosphatidylinositol 3,4,5-phosphate generation (10). As might be expected, ER and androgen receptor have been shown to directly couple with c-Src, whereas the progesterone receptor has not (25, 29), consistent with the notion that steroid hormone receptor-induced PI3-kinase activation is c-Src-dependent. In contrast, if steroid hormone receptors heteromultimerize, responses can be diversified. For example, PR and ER can associate in the absence of ligand; in this setting, either progestins or estrogens can rapidly trigger c-Src activation (25). Also, a ternary androgen receptor/ER/c-Src complex has been demonstrated, through an ER-pTyr$^{307}$/c-Src-SH2 and androgen receptor/c-Src-SH3 interaction (29). Whether ER-pTyr$^{307}$ is constitutively or inducibly (by estrogen) phosphorylated remains unclear.
The expectation is that those ER-dependent sequential c-Src/PI3-kinase/Akt activation events are rapidly catalyzed at the plasma membrane. This brings the focus back to that of a non-conventional, membrane-localized steroid hormone receptor-signaling pathway. There is an impressive and growing list of membrane steroid hormone-mediated responses in a variety of cells (9, 11–14, 47–55). We have recently taken advantage of the EAhy926 EC line, which, under the described culture conditions, does not express the 66-kDa, estrogen-responsive element-enhancing ER but does express a 46-kDa ER that is capable of transducing the signals we have described previously (9, 12). We are currently identifying the requirements for membrane localization and preferentially expressed forms of ER in vascular tissue, which are responsible for ligand-induced c-Src activation and consequent NO release. As we come closer to identifying the most proximal components of this signal transduction cascade, the feasibility of targeting reagents to positively modulate cardiovascular responses expands.

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