Published in final edited form as:

Trends Endocrinol Metab. 2002 October ; 13(8): 349–354.

Estrogen action and cytoplasmic signaling cascades. Part I: membrane-associated signaling complexes

James H. Segars and

Dept of Obstetrics and Gynecology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Rd, Bethesda, MD 20814, USA

Paul H. Driggers*

Building 10, Rm 9D-42, PREB, NICHD, NIH, 9000 Rockville Pike, Bethesda, MD 20892, USA

Abstract

Remarkable progress in recent years has suggested that estrogen action *in vivo* is complex and often involves activation of cytoplasmic signaling cascades in addition to genomic actions mediated directly through estrogen receptors α and β . Rather than a linear response mediated solely through estrogen-responsive DNA elements, *in vivo* estrogen might simultaneously activate distinct signaling cascades that function as networks to coordinate tissue responses to estrogen. This complex signaling system provides for exquisite control and plasticity of response to estrogen at the tissue level, and undoubtedly contributes to the remarkable tissue-specific responses to estrogens. In part I of this series, we summarize cytoplasmic signaling modules involving estrogen or estrogen receptors, with particular focus on recently described membrane-associated signaling complexes.

Knowledge of the molecular mechanism of estrogen action has evolved rapidly during the past two decades. It is now accepted that two proteins serve as receptors for 17β estradiol (E_2), estrogen receptors α and β (ER α , ER β). These receptors function as ligand-dependent transcription factors to increase gene transcription from promoters by direct binding of the receptor to specific DNA target sequences, designated estrogen response elements (EREs). Association of receptors with the gene transcription machinery involves essential coregulatory proteins that contribute to estrogen action and that can enhance or repress ER action (see Refs [1-3]). The importance of this signaling pathway *in vivo* has been substantiated using several approaches, including targeted gene disruption (knockout) in mice [4-8]. Within the ER proteins, there are two activating functions corresponding to the N-terminus (AF-1) or ligand-binding region (AF-2) of the molecule [9] (and Refs therein). In addition, ER function is modified by its phosphorylation [10,11]. The genomic actions of E_2 proceeding through augmentation or repression of ERE-containing promoters by ERs α and β have been designated the classic pathway of estrogen action [12]. In addition, ER acts through AP-1 and SP-1 to affect transcription.

*segarsj@mail.nih.gov.

In spite of the clarity with which the ER has been shown to act as a transcription factor, it has been apparent for several years that not all physiological effects of E₂ are accomplished through a direct effect on gene transcription [13]. Definition of the classic estrogen-signaling pathway highlighted the fact that, in many instances, another signaling pathway(s) involving cytoplasmic proteins, growth factors and/or membrane-initiated responses contributed to estrogen action (reviewed in Refs [14-18]). In Part I of this review, we focus on membrane-associated estrogen action, particularly cellular responses observed within minutes of estrogen exposure. Part II (to be published in the December issue of *TEM*) covers cytoplasmic cascades initiated by growth factors, and involvement of the ER in second messenger signaling cascades. This division is both artificial and inexact, because *in vivo* there is considerable overlap between the interaction of estrogen and growth factor signaling, and obviously growth factor-mediated responses begin at the plasma membrane. Nevertheless, some studies suggest that the two processes might be distinct, and recent developments are simply too vast to cover completely in a single review.

An immediate obstacle encountered in a review of cytoplasmic signaling modules of estrogen action is that there is no satisfactory system for classification of alternative pathways of estrogen signaling. The designation ‘nongenomic’ is not satisfactory, because some cytoplasmic signaling cascades involving estrogen produce increases in gene transcription, whereas other estrogen-mediated events are independent of transcription. Furthermore, cytoplasmic signaling cascades involving estrogen are in fact diverse, and general designations such as ‘nonclassical’ are inaccurate, because considerable detail is lost in this overgeneralization. Similarly, the concept of coupling between specific cytoplasmic modules and ER signaling is more accurate than ‘membrane or non-membrane’ nomenclature, because it is equally clear that not all cytoplasmic pathways leading to activation of the ER entail a membrane-based ER.

Different estrogen-dependent signaling pathways have been distinguished empirically based on variables including: subcellular location of the receptor complex, kinetics of the response, specific receptors and regions of receptor required for the response, proteins involved in the response, susceptibility to inhibition with pharmacological agents and the physiological and *in vivo* relevance of the pathway. For example, neuronal responses caused by ion fluxes are usually observed within 2 min. Cytoplasmic effects because of activation of kinases can occur within 2–5 min [19], and direct phosphorylation of either ER α or ER β is seen within hours, usually <4 h. By contrast, effects caused by gene transcription are initiated within 30–45 min [20], but require 4–8 h for translation and/or the effect to be measured [21]. Susceptibility of the effect to antiestrogens (such as ICI 162,780) might be a useful characteristic of the response. For purposes of discussion, responses can be grouped according to the apparent subcellular location of the receptor complex or second messenger signaling systems involved (Box 1).

Membrane-associated estrogen signaling pathways

Many estrogen-dependent neurological events are mediated by membrane-based ion fluxes, often involving Ca²⁺ or Ca²⁺-dependent K⁺ channels (reviewed in Ref. [22]). These actions are extremely rapid, and appear to involve membrane-associated signaling complexes that

are capable of responding to picomolar concentrations of estrogens. Kinetics of such responses are inconsistent with a mechanism requiring gene transcription. One such pathway is a direct effect of E₂ on the ion channels (within 5 min), but the components of this pathway have yet to be elucidated. A summary of the rapid actions of estrogen upon neurons and vasculature is beyond the focus of this discussion (reviewed in Refs [23,24]). Response to the membrane-impermeant compound estradiol-bovine serum albumin (E₂-BSA) has been used to define membrane-associated responses [25], but nonconjugated E₂ in some preparations might be a concern. Responses initiated with E₂-BSA might be functionally distinguished based on sensitivity to inhibition by antiestrogens.

Antiestrogen-sensitive membrane-associated signaling

Estrogen causes indirect modification of an existing ion channel. For example, in Guinea pig hypothalamic neurons, 20 nM E₂ induced rapid uncoupling of the μ -opioid receptor to a membrane K⁺ channel [26]. The effect was blocked by 2 nM of the antiestrogen, ICI164 384, but was not abolished with the protein synthesis inhibitor, cycloheximide, therefore protein synthesis was not required. Estrogen effects were mimicked by protein kinase A (PKA) activation and cyclic AMP (cAMP), but were blocked by Rp-cAMP (a nonhydrolysable cAMP analog) and KT5720, a staurosporine analog that inhibited PKA [26]. Because of the receptor pharmacology, authors concluded that physiological effects were not because of direct action on the μ -opioid receptor but rather a cellular pathway with a specific ER mediated via PKA [26]. The indirect effect was similar to effects of E₂ on GABA receptor, and repression of luteinizing hormone release [27]. In ovine endothelial cells, E₂ activated endothelial nitric oxide synthase (eNOS) activity, an effect blocked by the antiestrogen ICI 182 780 [28]. eNOS activation required the ligand-binding region of ER α , but did not require protein synthesis, and was inhibited by the compound PD98059, which inhibits mitogen-activated protein kinase kinase (MEK), and thus p42/44 mitogen-activated protein kinase (MAPK) activation [28]. In umbilical vein endothelial cells, rapid release of NO induced by E₂-BSA involved activation of guanylate cyclase, cGMP production and MAPK activation that was inhibited by ICI 182 780 [29]. The endothelial cells studied exhibited punctate membrane binding of E₂-BSA-FITC [29]. These examples suggest the existence of an antiestrogen-sensitive, cytoplasmic signaling cascade originating at the cell membrane that is capable of influencing several second messenger modules (Fig. 1).

Antiestrogen-insensitive estrogen membrane signaling

Antiestrogen-insensitive pathways initiated by E₂-BSA have been reported that involve MAPK and protein kinase C (PKC) [30]. E₂-BSA led to enhanced transcription of a murine c-fos reporter in SK-N-SH human neuroblastoma cells [30]. ICI 182 780 or tamoxifen did not block reporter activity, nor did E₂-BSA enhance an ERE-responsive reporter. The mechanism was reported to involve phosphorylation of MAPK [30]. In rat cortical explants, E₂ led to phosphorylation and activation of Erk1/2 within 5 min [31], an effect blocked by PD98059, but not inhibited by ICI 182 780. B-Raf kinase activity was increased and anti-ER antibodies precipitated a complex containing B-Raf, ER α and possibly heat shock protein (HSP)-90, suggesting that this membrane-based response might involve a multimeric complex containing B-Raf, a kinase that is immediately upstream of MEK1 [31]. Sylvia *et al.* [32] studied the response of resting and growing chondrocytes (GC) to E₂-BSA with

particular focus on defining the pathway involved. E₂-BSA caused a rapid increase in PKC, which was blocked by GDPβS, the phosphatidylinositol (PI)-specific inhibitor U73122, but not the PI 3-kinase (PI3K) inhibitor LY294022 or the antiestrogen ICI 182 780 [32]. The authors concluded that estrogen-induced PKC activity was dependent on a G protein-coupled phospholipase C through a membrane response that did not involve the classic ER [32]. Several other investigators have reported rapid signaling cascades triggered by estrogen that are insensitive to ICI 182 780 or estrogenic compounds, such as diethyl sulfate. αERKO (αER-knockout) mice showed preservation of rapid estrogen-associated membrane effects on neurons [33]; and rapid effects on kainate-induced events in the ERKO hippocampal neurons were the same as in wild-type mice. The effects were seen within 2–3 min, were not blocked by ICI 182 780, but were potentiated and mimicked by 8-bromo cAMP (an activator of PKA) and thus were cAMP-dependent [33]. The authors suggest that there might be a membrane-associated ER other than ERα [33]. Taken together, these reports provide strong evidence for estrogen-triggered responses not involving ERα/β, because known ERs are sensitive to inhibition by antiestrogens.

In addition to these two pathways, evidence from several groups indicates that antiestrogens can also stimulate rapid membrane-associated signaling, thus suggesting that a third category might be designated: antiestrogen-stimulated membrane events [34–38].

Is there a novel membrane-based estrogen receptor?

Although it is clear that membrane-based responses exist, the isolation, cloning and convincing *in vivo* localization of an endogenous membrane-based ER distinct from the classic ERα/β remains to be shown. Norfleet *et al.* [38] found that either E₂-BSA or a rabbit polyclonal Ab R4 directed against the hinge region of ERα caused prolactin release from rat pituitary GH3/B6/F10 cells. Punctate membrane staining was observed using Ab R4. Immunoprecipitation and immunoblot with H151, a mouse monoclonal antibody that was also directed against the hinge region of ERα, suggested a 66-kDa protein [38]. Investigators have reported purification of other putative membrane receptors. Immunoblot analysis of rat cortex using a monoclonal antibody directed against murine ER (Novacastra, UK) detected 112- and 116-kDa proteins [39]. Hormone-binding characteristics, sequence information and further characterization of the putative ERs were not described. More recently, two forms of ER, 67-(full-length) and 46-kDa, were copurified with 5' nucleotidase, a plasma membrane-marker enzyme [40]. The receptors were associated with MAPK and Akt and exhibited binding of E₂ with K_d to MCF-7 plasma membranes of 3.6×10^{-10} M [40]. Other investigators also reported a 46-kDa receptor [33,41,42]. Flouriot *et al.* [42] demonstrated that a 46-kDa ER form lacking the first 173 aa (AF-1 region) was derived from alternative splicing of the hERα gene and expressed in MCF-7 cells and extracts. This form inhibited function of the full-length ERα under conditions in which AF-1 responses predominated over AF-2 [42]. Furthermore, the targeting strategy used to generate αERKO mice [4] involved an exon that was skipped in generation of ERα46 transcript [42]: thus, ERα46 might be expressed in αERKO mice. Nevertheless, the relationship of smaller ER forms to membrane-associated estrogen responses remains unclear. However, all membrane forms described to date are related to ERα, and not β [38,40,42].

In agreement with the empirically observed distinction between antiestrogen-sensitive and insensitive signaling cascades, there is additional evidence to suggest that more than one type of membrane-based estrogen-responsive factor might exist. Although a distinct receptor remains to be characterized, antibodies directed against ER α have shown membrane binding in some laboratories, suggesting that one membrane-based receptor might be related to ER α . For example, membrane binding in pituitary cells was seen [43]. Dense membrane receptors were observed in hypothalamic neurons of guinea pig [26], and membrane-associated receptor forms were seen after transfection [44]. Membrane labeling in epithelial cells was also found [29]. In general, a greater density of receptors was present in nucleus and a lower density was seen in the cytoplasm. Immunohistochemistry revealed clustering of staining within the membrane, rather than an even distribution, and not all cells exhibited staining. By contrast, other groups report membrane-associated receptors that were neither ER α nor ER β [45,46]. IC-21 cells derived from mouse macrophages contained a G protein-coupled membrane-associated E₂ receptor that bound E₂-BSA-fluoresceine isothiocyanate (FITC) and became sequestered and internalized within one hour [46]. E₂-BSA binding did not colocalize with caveolin, and 1 nM E₂ was associated with a rapid rise in intracellular free Ca²⁺ that could not be inhibited by tamoxifen [46]. At present, there is no clear consensus, but it is possible that distinct membrane-based estrogen signaling pathways exist.

The physiological importance of membrane-based estrogen actions has been illustrated by several studies (reviewed in Ref. [22]). For instance, Razandi *et al.* [21] showed that membrane E₂ receptors might contribute to antiapoptotic effects of E₂, an effect accomplished by E₂-BSA and observed within 4 h. This time course was notably longer than for the kainate-induced effects mentioned above, and some studies of MAPK phosphorylation have shown effects within 2 min. In this system, E₂ inhibited and JNK stimulated apoptosis; ICI 182 780 inhibited the effect of estrogen [21]. Time-course studies showed that activation of an ERE-driven reporter construct was not detected until 8 h, thus the anti-apoptotic effects did not appear to require transcription [21]. The authors observed a 33–48% abolition of anti-apoptotic effects with PD98059 [21]; therefore the response was in part MEK 1-dependent. Also, Beyer and Karolczak demonstrated the role of membrane-associated estrogen action in dendritic growth involving phosphorylation of the cyclic AMP-responsive nuclear factor, cAMP-response element binding protein (CREB) [47]. In addition, nontranscriptional pathways have been suggested to be important for cell growth [48]. These observations, and many others not cited, suggest that membrane-based signaling pathways might impact important physiological processes.

Collectively, there is evidence for more than one membrane-based signaling pathway associated with estrogen action (Fig. 1). Although some differences could be attributed to cell-type specific responses, data from many laboratories suggest the existence of two functionally distinct membrane-associated pathways: one sensitive to antiestrogens and the second resistant. This difference argues for the existence of an unidentified estrogen-binding factor, because both ER α and ER β are sensitive to ICI 182 780, which blocks both AF-1 and AF-2-mediated responses. Membrane-based estrogen signaling has been shown to trigger distinct signaling cascades involving cAMP [49], release of intracellular Ca²⁺ stores [47], Ca²⁺ fluxes [50], MAPK [30] and phosphorylation of CREB [51]. The compelling

demonstration of immediate membrane-based responses, albeit involving diverse second messenger cascades, raises the possibility that estrogen might pass through the outer cell membrane by a process of facilitated transfer using a distinct carrier system. Reports of a 46-kDa ER are intriguing, but current evidence suggests that this form of ER alone would not be sufficient to account for the observed heterogeneity in membrane-associated estrogen effects.

Estrogen coupling to eNOS might involve $G_{\alpha i}$, p85 PI3K, or caveolin-1

What are the specific molecular mechanisms that are responsible for the distinct signaling pathways? One emerging membrane-associated cytoplasmic signaling cascade involves caveolae, flask-shaped invaginations in the plasma membrane [52], and the associated proteins, NOS, p85 and caveolin-1 (for review of estrogen and NO see Ref. [53]). Whether this signaling complex accounts for some or all of the pathways in the preceding paragraphs is not yet clear. Schlegel *et al.* [54] demonstrated that ER α bound directly to caveolin-1, a protein component of caveolae. Furthermore, overexpression of caveolin-1 caused ligand-independent nuclear translocation of ER α and increased ligand-dependent and independent gene activity in MCF-7 cells [54]. In bovine aortic cells, E₂ led to a rapid (<5 min) increase in NO production cells without an increase in eNOS [55]. The response involved a rapid intracellular increase in cytosolic Ca²⁺ and the authors suggested that ER α was a component of caveolae [55], although localization of ER α to caveolin was not shown. Notably, eNOS has been localized to caveolae, and ER α binding to caveolin-1 and -2 has been reported [56]. In addition, E₂ rapidly stimulated association of ER-caveolin in vascular smooth muscle cells (VSMCs), but inhibited the association in MCF-7 cells [56]. Moreover, in MCF-7 cells overexpression of caveolin-1 reduced E₂ stimulation of MAPK activation, thus suggesting cell-specific signaling modules and that Erk-related E₂ effects might be distinct from caveolin-mediated estrogen signaling [56].

Specifically, in a human endothelial cell line E₂ led to eNOS activation via phosphorylation on the crucial 473 Ser residue of Akt (Akt is a PI3K effector kinase) which, in turn, led to phosphorylation on Ser 1177 of eNOS [57]. The authors found that activation required PI3K, because either LY294002, an inhibitor of PI3K, or a dominant-negative-kinase-deficient Akt mutant blocked the effect [57]. Activation by E₂ was found to occur within 5 min. Simoncini *et al.* [58] extended these findings to show that E₂ stimulated eNOS through a direct association of ER α with the P85 subunit of PI3K. The association was ligand-dependent and was not blocked by PD98059, but was blocked by ICI 182 780 and wortmannin [58]. The involvement of PI3K/Akt in E₂ signaling was investigated further by Wycoff *et al.* [59] who found E₂ activated NOS similar to acetylcholine through a pertussis toxin-sensitive pathway in endothelial cells. Interestingly, anti-ER antibodies (AER 320) immunoprecipitated ER α with $G_{\alpha i}$, but not other G protein subunits, and interaction of ER α with $G_{\alpha i}$ was blocked by ICI 182 780 [59]. The effect was reduced by RGS4, a compound that accelerated GTPase activity in these cells [59]. More specific studies of requirements for the interaction or dissection of the complex were not reported. The authors suggested a multiprotein complex and that the interaction was possibly indirect [59]. It is possible that this complex influences PI3K/Akt activity. *In vivo*, estrogen decreases the number of caveolae in uterine smooth muscle, an effect that was blocked by ICI 182 780 [60]. In

support of a relationship between E₂ and caveolin, E₂ differently influenced caveolin production in MCF-7 cells and VSMCs [56].

The PI3K/Akt pathway of estrogen action might antagonize tamoxifen-induced apoptosis in MCF-7 cells [61]. PI3K activated AF-1 and AF-2 of ER α in the absence of ligand, but Akt activated by AF-1 only [61]. Akt phosphorylation involved Ser 167 ER α and mutation of the residue to Ala destroyed the effect [61]. PTEN (a negative regulator of PI3K) and a catalytically inactive Akt mutant reduced PI3K-induced effects, suggesting a link between PI3K/Akt and hormone-independent activation of ER α [61]. Taken together, observations from several laboratories argue for a cytoplasmic signaling unit associated with caveolae in a multiprotein complex localized to the cell membrane, possibly including caveolin-1, the p85 subunit of PI3K, and G α_i (Fig. 2). The precise relationship of this putative complex to larger caveolin complexes or the proposed HSP-90–ER–B-Raf complexes remains unclear, but HSP-90 also binds to and activates eNOS [62]. Furthermore, estrogen stimulates HSP-90 binding to eNOS [63]; thus, it is conceivable that the B-Raf–HSP-90 module and the caveolin-complex pathways might be interrelated. Additional studies are needed to clarify the relationship of this pathway to membrane-associated estrogen signaling.

Conclusions and perspectives

Although current understanding of cytoplasmic signaling networks involving estrogen is incomplete, recent studies have begun to define distinct signaling complexes that appear to be involved in membrane-associated signaling by E₂. Empirically, at least two pathways can be distinguished, one that is sensitive to inhibition by antiestrogens, and the other that is resistant. The description of antiestrogen-insensitive responses raises the possibility of another estrogen-binding protein (or factor).

Distinct signaling cascades involving specific complexes of cytoplasmic proteins have been shown to orchestrate estrogen action. In Part I of this series, we have reviewed recent reports of involvement of ER and a caveolin-associated signaling complex. Given the complex nature of these cytoplasmic modules, experimental results must be interpreted with caution. For example, overexpression of factors might give misleading information regarding the nature of signaling, because an overabundance of a factor might lead to compensatory effects not relevant *in vivo*. Much remains to be learned about the mechanisms responsible for specificity and the role of cytoplasmic proteins in signal transduction for estrogens.

Acknowledgments

We thank George Anderson for his help in the preparation of this review and Domenica Rubino and John Wu for their critical reading of it. P.H.D. is supported, in part, by a grant from the DOD. We also thank George Chrousos and William Haffner for their support and assistance.

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Box 1. Empirical classification of cytoplasmic signaling involving estrogen or ERs

1. Membrane-based ion fluxes

Direct action
 Indirect action (modification of existing ion channel)
 Antiestrogen-sensitive mechanism
 Antiestrogen-insensitive mechanism
 Ca^{2+}
 K^{+}
 Intracellular versus extracellular ion pools

2. Second messenger systems

cAMP
 PKA
 P44/42 MAPK
 P38 MAPK
 PI3/Akt
 NOS
 G protein ($\text{G}_{\alpha i}$)
 DAG
 cGMP

3. Modification of existing membrane receptors

IGF-I receptor
 EGF receptor

4. Indirect effects of ERs on non-ERE-containing promoters

Brn 3a and Brn 3b
 Stat 5b
 NF- κ B
 Fos/Jun
 SP1

5. Modification of transcription factors

Abbreviations: cAMP, cyclic AMP; cGMP, cyclic GMP; DAG, diacylglycerol; EGF, epidermal growth factor; ERE, estrogen response element; IGF I, insulin-

like growth factor-I; MAPK, mitogen-activated protein kinase; NF- κ B, nuclear factor kappa B; NOS, nitric oxide synthase; PI3K, phosphatidylinositol-3-OH kinase; PKA, protein kinase A; Stat 5b, signal transducers and activators of transcription.

Fig. 1.

Functionally defined signaling modules involving a putative membrane-based estrogen-responsive factor. (a) Three antiestrogen-sensitive pathways that are inhibited by ICI 182 780. (b) Three membrane-based signaling pathways that are not affected by antiestrogens. Signaling modules are cell-type specific. Question marks indicate the possibility of another membrane-based receptor. Abbreviations: eNOS, endothelial nitric oxide synthase; ER, estrogen receptor; HSP, heat shock protein; MEK, mitogen-activated protein kinase kinase; Pi-PLC, G protein-dependent phosphatidylinositol-specific phospholipase C; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C.

Fig. 2.

Caveolin-based estrogen signaling. ERs are shown in a complex with caveolin-1, a protein component of caveoli. Results from several groups [49-51] suggest a complex with ER α possibly involving G α_i , p85 subunit of PI3K [52-54], and caveolin-1, which affects eNOS activation. HSP90 has also been shown to bind ER α , and affect eNOS activity. Modules appear to be cell-type specific. Abbreviations: eNOS, endothelial nitric oxide synthase; ER, estrogen receptor; GTP, guanosine triphosphate; HSP, heat shock protein; PI3K, phosphatidylinositol 3-kinase.