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Membrane-initiated Actions of Estrogen on the Endothelium

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Abstract

Estrogen-induced rapid, membrane-initiated activation of numerous signal transduction cascades has been shown in animal, cellular and molecular vascular studies, which support the favorable effects of estrogen on vascular structure and function. These effects are mediated by distinct forms of estrogen receptor (ER) α . This includes estrogen-stimulated, rapid activation of endothelial nitric oxide synthase (eNOS), resulting in elaboration of the athero-protective, angiogenesis-promoting product nitric oxide (NO). An N-terminus truncated short isoform of ER α , ER46, plays a critical role in membrane-initiated, rapid responses to 17 β -estradiol (E2) in human endothelial cells (ECs). We have proposed a ER46-centered, eNOS-activating molecular complex in human EC caveolar membranes, containing c-Src, phosphatidylinositol 3-Kinase (PI3K), Akt and eNOS. In this review, we describe estrogen-induced, rapid, non-genomic actions in the endothelium.

Keywords

Estrogen; ER46; eNOS

1. Introduction

Steroid hormones modulate a large number of biological activities in mammals, affecting gene expression, growth and physiology in all organ systems. The cardiovascular system is an important estrogen target. A cardiovascular protective effect of estrogen has been suspected for many years, largely on the basis of the lower incidence of coronary heart disease (CHD) in premenopausal women, relative to that in age-matched males, as well as on the basis of several observational studies (Stamper and Colditz, 1991; Grady et al., 1992). The most basic actions of estrogens were recognized in the 1950s. Jensen and Jacobson concluded that the biological effects of estrogen are mediated by a receptor protein (Jensen and Jacobsen, 1962). The first recognized steroid hormone receptor was an estradiol binding protein (Jensen, 1965), following which Toft and Gorski isolated and characterized an estrogen receptor (ER) from rat uterus (Toft and Gorski, 1966). Pietras and Szego described membrane binding sites for estrogen in endometrial cells (Pietras and Szego, 1977). They partially purified and initially characterized ERs from hepatocyte plasma membranes (Pietras and Szego, 1980). Since that time, estrogen-induced membrane-initiated signaling cascades have been defined. Of note, the

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rapid cellular action of steroid hormones was first reported in 1942 (Selye, 1942). In this review, we will discuss the importance of ER, especially ER46, in rapid, membrane-initiated effects of estrogen and its signaling in the endothelium.

2. Estrogen receptor (ER) and ER46

There are 2 known ER genes encoding classical ERs, ER α and ER β , initially isolated from breast cancer MCF cells and rat prostate, respectively (Green et al., 1986; Greene et al., 1986; Kuiper et al., 1995). These two ERs share high sequence homology except in their amino terminus, bear similar affinities for estrogen and bind the same DNA response elements. A G protein-coupled 7-transmembrane receptor, GPR30, unrelated to classical ER structure but with high affinity to E2 has also been recently described (Revankar et al., 2005). Classical ERs are members of the nuclear receptor transcription factor superfamily. In genomic estrogen action, binding of estrogen to the ER ligand binding domain (LBD) induces a receptor conformational modification and dimerization, exposure of nuclear localization sequences (NLS), and nuclear translocation, with consequent binding to estrogen response elements (EREs) on the promoter or enhancer regions of target genes (Truss and Beato, 1993). There is additional modulation of transcription through ligand-bound ER interactions with other transacting factors, i.e., not through direct DNA binding. The array of resultant mRNAs and their encoded protein expression constitutes physiological estrogen responses. In addition, there are rapid estrogen responses that occur within seconds to minutes, independent of transcription without changes in gene expression. These rapid responses to estrogen are mediated by forms of ER α or by other membrane-associated estrogen-binding proteins in ER negative cells.

ER α and ER β are both expressed in endothelial cells (ECs) and vascular smooth muscle cells, mediating both the rapid, membrane-initiated and genomic cardiovascular effects of estrogens. Similar ER α and ER β protein expression levels are expressed in the carotid arteries of young and aged rats (Miller et al., 2007). However, reduced ER expression has been detected in human atherosclerotic arteries (Losordo et al., 1994; Nakamura et al., 2004). Furthermore, there are a variety of ER splice variants, giving rise to a substantial heterogeneity of ER expression which is, in part, tissue-specific (Flouriot et al., 2000; Figtree et al., 2003). We have demonstrated the abundance of an N-terminus (A/B or AF-1)-deleted ER α splice isoform, ER46, in ECs, which is an efficient transducer of membrane-initiated responses (Haynes et al., 2003; Li et al., 2003). This definition of the ER α splice isoform, ER46, in ECs, opened a new chapter on estrogen effects in the vasculature.

3. Effects of estrogen on the vascular system

Estrogen binds to vascular endothelial and smooth muscle cells with high affinity (Grady et al., 1992; Farhat et al., 1996). Estrogen has numerous nuclear-initiated genomic effects on ECs, including the repression of athero-promoting and the induction of athero-protective genes (Caulin-Glaser et al., 1996; Mendelsohn and Karas, 1999). Estrogen also stimulates rapid activation of vascular endothelial nitric oxide synthase (eNOS) and rapid vasodilation mediated by ERs (Chen et al., 1999; Mendelsohn and Karas, 1999). eNOS is the classic “vasculoprotective” NOS isoform and is expressed in ECs, cardiac myocytes and blood platelets (Govers and Rabelink, 2001). Here, we discuss the ER-mediated, nongenomic endothelial responses to estrogen, resulting in NO production through rapid eNOS activation.

Endothelial homeostasis is critical to vascular health. The constitutive and induced production of NO is a key, positive regulatory factor in this homeostatic state, as NO has potent anti-thrombotic, anti-leukocyte adhesive and anti-smooth muscle cell proliferative properties (Moncada and Higgs, 1993). Endothelial dysfunction, largely defined as a lack of vasodilation to NO-inducing stimuli, correlates with an increased risk of adverse cardiovascular events

(Harrison, 1997). ER α and ER β are both expressed in ECs and vascular smooth muscle cells, mediating both the rapid, membrane-initiated and genomic cardiovascular effects of estrogens. E2-stimulated, enhanced NO production in murine aorta persists in ER β knockout (ER β KO) but not in ER α knockout (ER α KO) mice (Darblade et al., 2002). We and others have utilized murine models to assess vascular responses to 17 β -estradiol (E2) (Darblade et al., 2002; Florian et al., 2004; Guo et al., 2005; Li et al., 2007). Physiologically functional short ER α isoforms were demonstrated in ER α KO mice. The initially confounding findings that both ER β KO and ER α KO mice retain protective responses to E2 were eventually explained by the retention of ER α splice forms ER55 and ER46 in the exon 1-targeted, ER α KO mice (Iafrati et al., 1997; Karas et al., 1999; Pare et al., 2002). The exon 2-targeted, complete ER α KO (ER α KO_{Strasbourg}) mice lost the E2-induced re-endothelialization observed in wild-type mice (Bouchet et al., 2001). It was then understood that products of the ER α gene conferred this form of vascular protection, and that ER α splice variants are capable of mediating these favorable responses. It was subsequently shown that both full-length ER α and the N-terminus truncated ER46 co-localize with eNOS in plasma membranes, and that E2 induces rapid eNOS activation. ER46 mediates E2-stimulated eNOS activation more efficiently, whereas E2-stimulated genomic responses favor ER α over ER46 (Figtree et al., 2003; Li et al., 2003). A recent study using ER α knock-in mutant mice supports the importance of ER α splice variants. In this work, a mutant ER α , with deletion of the 441 nt of exon 1 corresponding to amino acids 2–148, was expressed in ER α KO mice. The study demonstrates that the ER α N-terminal AF-1 domain is dispensable for the major vasculoprotective effects of E2, such as basal endothelial NO production, acceleration of reendothelialization and atheroprotection (Billon-Galés et al., 2009).

E2-induced vascular eNOS activation and NO production are Akt-dependent (Florian et al., 2004). Endothelial- (i.e., eNOS-) dependent, E2-enhanced vasodilation has been shown to require ER triggering of the ERK/MAPK, PI3K (Guo et al., 2005) and the non-receptor tyrosine kinase c-Src pathways (Li et al., 2007). We have demonstrated the loss of estrogen-stimulated, endothelial- and NO-dependent vasodilation of phenylephrine (PE) precontracted aorta isolated from c-Src gene deleted mice (Li et al., 2007).

The aforementioned, non-classical ER, GPR30, is variably involved in normal estrogenic responses. For example, GPR30 is required for normal growth plate responses to estrogen. However, it is not responsible for estrogen-stimulated increases in uterine weight, thymus weight, and fat mass (Isensee et al., 2008; Windahl et al., 2009). Recently, a selective GPR30 agonist has been used to demonstrate vasodilating responses in rat mesenteric and mouse carotid arteries (Haas et al., 2009). As with classical ERs, this suggests that GPR30 may play a cardiovascular protective role.

The loss of gender-based cardiovascular protection in postmenopausal women has been attributed to waning estrogen levels. However, it is possible that aging results in a fundamental change in vascular cell responses to estrogen. E2-induced inhibition of inflammatory cell recruitment and neointima formation is lost in injured carotids of aged rats, compared to young controls, despite similar ER α and ER β expression levels (Miller et al., 2007). Aged rat thoracic aortas lose vasodilating responses both to acetylcholine and estrogen, at least in part due to a significant reduction in soluble guanylyl cyclase (sGC) levels. This loss of sGC could be prevented by estrogen (Stice et al., 2009). An additional role for estrogen, as relates to aging, is in mitochondrial responsiveness. ERs expressed in mitochondria, and are thought to be responsible for a ligand-induced reduction in reactive oxygen species (Razmara et al., 2008). As mitochondrial biogenesis is reduced in aging, it is possible that estrogen is a less potent anti-oxidant trigger (Razmara et al., 2008), thereby creating a vascular environment known to be pathology-prone. This may represent an important component of aging-related vascular disease, and is an active area of investigation.

4. Localization of ERs at the plasma membrane

Indirect evidence for plasma membrane-associated ERs by cell imaging techniques initially supported the concept of rapid membrane-initiated estrogen responses (Pappas et al., 1995; Ropero et al., 2002; Li et al., 2003). ER localization at the plasma membrane is dependent on posttranslational modification and association with other proteins. We and others have demonstrated that ER palmitoylation is critical for plasma membrane localization (Li et al., 2003; Acconcia et al., 2004; Pedram et al., 2007). A critical S-palmitoylation site of the ER α , Cys447, supports ER α plasma membrane targeting (Acconcia et al., 2005), and a highly conserved ER α sequence (445–453 aa) is required for plasma membrane localization via palmitoylation (Pedram et al., 2007).

Lipid modifications of proteins, including palmitoylation and myristoylation, affect not only the localization of proteins to hydrophobic domains, but also interactions of proteins targeted to the plasma membrane. ER is a part of a molecular complex at the plasma membrane which, dependent on the cell type and context, includes c-Src (Castoria et al., 2001), caveolin-1 (Razandi et al., 2003), Shc (Song et al., 2002), the regulatory subunit of PI3K (p85) (Simoncini et al., 2000), multiple receptor tyrosine kinases (Levin, 2005; Song et al., 2004), as well as 2 G-protein (G α) isoforms (Razandi et al., 1999; Razandi et al., 2004). Several features of c-Src, caveolin-1, eNOS and ER membrane targeting have been defined (Russell et al., 2000; Haynes et al., 2000; Haynes et al., 2003; Li et al., 2003; Li et al., 2007). c-Src membrane targeting is known to require myristoylation on the Gly2 residue. Overexpression of a myristoylation-deficient c-Src mutant (SrcG2A) in ER46-transfected COS-7 cells dramatically diminishes both c-Src and ER46 plasma membrane localization, demonstrating their interdependence for membrane targeting (Li et al., 2007). In E2-induced activation of ECs, c-Src directs the generation of a functional signaling complex comprised of ER46, c-Src and p85 (Haynes et al., 2003). The colocalization and association of ER with caveolin-1 has been demonstrated in human ECs, cancer cells and myometrial cells (Li et al., 2003; Acconcia et al., 2005; Kiss et al., 2005). Caveolin-1 is a caveolar structural protein with a long intramembrane domain and multiple palmitoylation sites, which directs caveolae targeting of multiple signaling molecules (Kim et al., 1999; Kelly and Wagner, 1999; Lee et al., 2001; Chambliss et al., 2002). The aforementioned N-terminus-deleted ER α splice isoform, ER46, has been identified in human ECs, osteoblasts and MCF-7 cells (Flouriot et al., 2000; Denger et al., 2001; Li et al., 2003). This isoform is efficiently targeted to human EC caveolae at the plasma membrane, and such lipid raft targeting is augmented by E2 stimulation (Li et al., 2003). Paradoxically, E2 stimulation in cervical cancer cells can reduce ER α palmitoylation levels and consequently reduce the ER α -caveolin-1 complexes (Acconcia et al., 2005). *In vivo* studies show that E2-induced Src activation can promote caveolin-1 phosphorylation and diminished levels of plasma membrane associated caveolin-1 (Kiss et al., 2005). ER α Ser522 is a critical caveolin-1 interaction residue, and the deletion of the caveolin-1 scaffolding domain (60–100 aa) prevents ER α plasma membrane localization (Razandi et al., 2003). Thus, palmitoylation and caveolin-1 interactions are required for ER membrane localization. The effect of estrogen on these critical molecular phenomena is still being elucidated and likely cell type-specific.

5. Estrogen and rapid membrane-initiated signaling

The most studied vascular response to E2 is eNOS activation resulting in NO release. Caveolar localization of eNOS is required for its activity (Shaul et al., 1996; Govers and Rabelink, 2001). Myristoylation and palmitoylation of eNOS result in binding to endothelial caveolin-1 (García-Cardena et al., 1997). eNOS activity is augmented by estrogen in caveolae, but not in non-caveolae, subcellular fractions. This E2-stimulated event is inhibited by the ER antagonist ICI 182,780 (Chambliss et al., 2000). In this work, ER α levels were much greater in these caveolae fractions. We have found the same preferential localization of ER46 in endothelial

caveolae fractions, especially in the presence of estrogen (Li et al., 2003). It is now clear that the ER-mediated activation of eNOS is caveolin-1-dependent, and that the caveola is the signaling organelle in which these activation events occur. We have proposed that a caveolae-localized, ER46-centered, multi-molecular complex is critical in rapid eNOS activation and endothelial NO release (Figure 1; Kim and Bender, 2005).

Other laboratories have shown that E2-stimulated eNOS activation and NO production are regulated by Akt (Florian et al., 2004), and that ER-mediated eNOS activation can be regulated by ERK/MAPK signaling as well (Guo et al., 2005; Chen et al., 1999; Chambliss and Shaul, 2002). Most relevant is the E2-induced, rapid phosphorylation of eNOS on the Ser1177 activation site and the consequent augmentation of NO release in EC (Russell et al., 2000). Our findings support that the estrogen-induced rapid membrane-initiated eNOS activation is mediated by a sequential c-Src/PI3K/Akt cascade in EC (Figure 2; Haynes et al., 2003). This rapid estrogen signaling involves the interaction of ER with PI3K in a ligand-dependent manner (Simoncini et al., 2000; Haynes et al., 2003). Posttranslational modifications of ER and c-Src are important for protein-protein interaction and eNOS activation. c-Src-dependent E2-induced phosphorylation of ER46, likely of Tyr537, leads to an amplified c-Src SH2 domain interaction, resulting in eNOS activation and NO release. A myristoylation-deficient c-Src mutant (Gly2Ala) does not support membrane-impermeant E2-stimulated phosphorylation of the critical c-Src Tyr419 activation site, nor eNOS activation (Ser1177 phosphorylation or enzymatic activation) (Li et al., 2007).

In addition to eNOS activation with consequent NO production, E2 induces cyclooxygenase-2 and rapidly stimulates the secretion of prostaglandins PGI₂ and PGE₂ in human ECs (Pedram et al., 2002). One of the endothelium-derived relaxing factors, endothelium-derived hyperpolarizing factor (EDHF) induced by estrogen stimulates endothelium-dependent vasodilation in reproductive vessels, but not in nonreproductive vessels (Rahimian et al., 2004; Burger et al., 2009). Estrogen also enhances NO bioavailability mediated by the inhibition of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase expression, thus limiting the generation of superoxide (O₂⁻) and peroxynitrite (Traupe et al., 2007).

6. Selective estrogen receptor modulators (SERMs)

The potentially favorable biological effects of estrogen and ER engagement in the vascular system are recognized, although several randomized clinical trials, including the large Women's Health Initiative (WHI) trial, have either failed to find a benefit, or even shown a detriment, of hormone replacement therapy on the incidence of cardiovascular events, in postmenopausal women (Grodstein et al., 1997; Hulley et al., 1998; Viscoli et al., 2001; Rossouw et al., 2002; Yeboah et al., 2007). Amidst ongoing controversies in hormone replacement therapy (HRT), these molecular and cellular data, defining favorable estrogenic effects on the endothelium, provide a strong impetus to resolve clinical questions regarding estrogen's role in vascular protection. This had led to a search for selective estrogen receptor modulators (SERMs) that have a beneficial effect profile on the endothelium. SERMs can be tissue-specific agonists and/or antagonists of estrogen action.

Tamoxifen, a first generation SERM, increases eNOS phosphorylation and induces endothelial-dependent relaxation in porcine coronary arteries (Leung et al., 2006). Raloxifene activates eNOS and enhances NO release in a PI3K-, AKT-, and, variably, ERK-dependent manner (Hisamoto et al., 2001; Simoncini et al., 2002a). Raloxifene stimulates endothelium-dependent relaxation through up-regulation of eNOS expression and activity in porcine coronary arteries *ex vivo* (Leung et al., 2007). Raloxifene also protects the endothelium against oxidative stress, both *in vitro* and *in vivo* (Wassmann et al., 2002; Wong et al., 2008). However, the Raloxifene Use for the Heart (RUTH) clinical trial failed to demonstrate a reduction in

CHD risk. As with E2 and cardiovascular disease prevention, the discrepancy between favorable vascular effects *in vitro* and beneficial effects *in vivo* requires further elucidation (Barrett-Connor et al., 2006). A fourth generation SERM, acolbifene, also rapidly activates eNOS in a PI3K/AKT-dependent fashion (Simoncini et al., 2002b). Recently recovered endogenous SERM, 27-hydroxycholesterol (27HC), impairs estrogen-induced rat aorta vasorelaxation by inhibiting both transcription- and non-transcription-mediated, estrogen-dependent NO production. 27HC also represses E2-stimulated carotid artery reendothelialization (Umetani et al., 2007). In contrast, 27HC is an estrogenic agonist in non-vascular tissue (DuSell et al., 2008). There remains a great deal of interest in defining SERMs that selectively have beneficial effects on the endothelium.

7. Conclusions

The estrogen story, with regard to cardiovascular disease prevention, continues to evolve. Simply stated, the numerous favorable effects of estrogen on the endothelium at the cellular and molecular level, many manifested through rapid signaling responses and eNOS activation, should translate, at some level, to beneficial clinical effects. Large, randomized clinical trials have generally studied postmenopausal women, likely with established atherosclerotic disease, thereby obscuring any of estrogen's benefit on atherogenesis. Furthermore, there are very significant unfavorable effects, many of which relate to cell proliferation, most notably of epithelial tissues. It is the hope that, in balance, ER targeting could be performed in such a way that would include rapid, membrane-initiated responses in endothelial cells, while avoiding the aforementioned genomic responses in epithelial tissues. We also now recognize that the distinction between non-genomic, membrane-initiated and genomic, nuclear-initiated signaling is greatly over-simplified, and that much crosstalk between these mechanisms exists. It is therefore imperative that the molecular details of ER biology, including splice isoform expression, membrane vs. nuclear targeting and protein-protein interactions are defined. Such definitions will also provide greater bases for SERM development and use, an evolving field with great promise. Thus, the merging of better designed clinical trials, definition of precise molecular details, and development of very specific pharmaceuticals will undoubtedly bring us much closer to effective therapeutics in hormone therapy.

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Figure 1.

ER46-centered molecular complex in caveolae including c-Src, PI3K, Akt, Hsp90 and eNOS. This complex initiates rapid responses to estrogen at the membrane in human ECs, leading to eNOS activation and NO production. Molecular features of ER46 with Cav-1 and c-Src shown at the bottom. These include an ER palmitoylation site, a c-Src myristoylation site, and a caveolin-1 interaction site, all critical for membrane localization. Reprinted with permission (Kim and Bender, 2005).

Figure 2. Membrane-initiated, rapid responses to estrogen in human ECs, leading to eNOS activation and NO release.