Non-nuclear Actions of Estrogen: New Targets for Prevention and Treatment of Cardiovascular Disease

Karen J. Ho and **James K. Liao**
The Cardiovascular Division, Department of Medicine, Brigham and Women's Hospital, and Harvard Medical School, Cambridge, MA 02139, USA

Abstract

Gender-based differences in the incidence of hypertensive and coronary artery disease, the development of atherosclerosis, and myocardial remodeling after infarction are attributable to the indirect effect of estrogen on risk factor profiles such as cholesterol levels, glucose metabolism, and insulin levels. More recent evidence, however, suggests that activated estrogen receptor (ER) mediates signaling cascades that culminate in direct protective effects such as vasodilation, inhibition of response to vessel injury, limiting myocardial injury after infarction, and attenuating cardiac hypertrophy. Although the ER is usually thought of as a ligand-dependent transcription factor, it can also rapidly mobilize signals at the plasma membrane and in the cytoplasm. Thus, a greater understanding of ER function and regulation may lead to the development of highly specific therapeutics that mediate the prevention and treatment of cardiovascular diseases.

INTRODUCTION

Gender-based differences in the incidence of hypertensive and coronary artery disease, the development of atherosclerosis, and myocardial remodeling after infarction are attributable to the indirect effect of estrogen on risk factor profiles, such as cholesterol levels, glucose metabolism, and insulin levels (1–3), as well as its direct effects on the myocardium, vascular smooth muscle and endothelium. Although estrogen receptor (ER) is typically thought of as a ligand-dependent transcription factor, it also modulates the activity of intracellular second messengers and membrane-associated signaling complexes. In the heart and vasculature, these non-nuclear signaling pathways mediate rapid vasodilation (4), inhibition of response to vessel injury (5–10), reduction in myocardial injury after infarction (11,12), and attenuation of cardiac hypertrophy (13,14).

ESTROGEN RECEPTOR STRUCTURE AND FUNCTION

Both subtypes of ER, ERα and ERβ, are members of the nuclear receptor superfamily (15, 16). They are synthesized from separate genes and are structurally and functionally distinct. Classically, ER regulates gene expression in target tissues in a ligand-dependent manner: the binding of estradiol (E2) releases ER from an inhibitory complex and allows for receptor homodimerization and translocation into the nucleus (1,2,17). The receptor then binds a palindromic estrogen response element (ERE) located in the promoter region of target genes. The concerted actions of the ligand-independent activation function domain (AF-1) in the N terminus (Figure 1) and the ligand-dependent AF-2 region in the hormone-binding domain lead to the recruitment of tissue-, cell-, and promoter-specific co-regulator complexes to the ERE, resulting in transactivation or transrepression (18,19).

Gene deletion or mutation studies have underlined the importance of ER in cardiovascular physiology (20). Early studies of ovariectomized mice demonstrated that E2 inhibits the proliferation of intimal and medial vascular smooth muscle (5), suggesting a direct protective

effect of estrogen on endothelium and vascular smooth muscle cells (VSMCs). In ERα and ERβ double-knockout mice, however, E2 inhibits VSMC proliferation but not medial thickening, suggesting that a leakily expressed splice-variant of ERα could mediate partial protection (21,22). The more recent production of complete ERα-null mice (23), which exhibit increased medial area, VSMC proliferation, and deposition of proteoglycans in response to vascular injury, has confirmed the role of ERα in vascular protection (24). The effects also extend to the myocardium. For example, ERα-deficient hearts subjected to whole-organ ischemia and reperfusion (25) exhibit greater ischemia and higher incidence of arrhythmias than that observed in wild-type hearts. The process may involve nitric oxide (NO), which ameliorates coronary dysfunction and reduces tissue edema by decreasing microvascular permeability, because ERα-deficient hearts also demonstrate decreased NO release.

In 1975, Pietras and Szego first described membrane binding sites for estrogen and described a non-genomic mechanism for calcium influx in endometrial cells (26). More recent studies have added to our current understanding of the highly tissue-specific, non-nuclear ERα signaling network. Though there is also evidence that ERβ has an important function in the vasculature (27,28), we focus on ERα because of the greater number of observations that have been made. Defining the cascades through which ERα elicits its pleiotropic cellular effects and understanding the dysregulation of the network in disease states promises to uncover novel targets for pharmacological intervention.

NON-NUCLEAR ACTIVITY OF ESTROGEN

Estrogenic transcription-dependent effects, such as those that contribute prominently in organogenesis and function of the reproductive system, become evident hours after stimulation. Non-nuclear (alternatively referred to as "non-transcriptional" or "non-genomic") estrogenic action peaks minutes after stimulation in multiple cell types. Other characteristics include immunity to inhibitors of DNA transcription or protein synthesis (actinomycin D or cycloheximide) and recruitment of membrane or cytosol-localized signaling components. These include the second messengers calcium and nitric oxide (NO), receptor tyrosine kinases including the epidermal growth factor receptor (EGFR) and insulin-like growth factor-1 (IGF-1) receptor (IGF1R), G protein coupled receptors (GPCRs), and protein kinases including phosphatidylinositol-3′ kinase (PI3K), the serine-threonine kinase Akt, mitogen-activated protein kinase (MAPK) family members, the non-receptor tyrosine kinase Src, and protein kinases A and C (PKA and PKC, respectively) (Figure 2) (for reviews see 17,29,30).

SIGNALING CASCADES ACTIVATED BY ESTROGEN

The PI3K-Akt signaling cascade is one downstream target of non-nuclear estrogenic signaling (31–33). In the vasculature, short-term exposure to E2 leads to NO-dependent vasodilation (34). The secretion of NO by healthy vessels relaxes smooth muscle cells and inhibits platelet activation in a cyclic guanosine 3′, 5′-mono-phosphate (cGMP)-dependent mechanism. In cultured endothelial cells, estrogen enhances NO release within minutes without altering expression of endothelial nitric oxide synthase (eNOS) (33,35). E2 activates eNOS activity in a biphasic manner through MAPK and PI3K-Akt pathways, leading to enhanced NO release (32). Myocardial protection by high-dose corticosteroids during ischemia-reperfusion injury also appears to be mediated by PI3K-Akt (36). In both cases, ERα and glucocorticoid receptors activate PI3K by associating with the p85α regulatory subunit in a ligand-dependent manner (32). Furthermore, the 90-kDa heat shock protein (HSP90) interacts with both eNOS and Akt and modulates eNOS activity by acting as a scaffold to regulate Akt-dependent phosphorylation of eNOS (37).

MAPK family members are common targets of non-nuclear estrogenic signaling. Induction of eNOS and inducible NOS (iNOS) expression in cardiac myocytes is blocked by the MAPK

inhibitor PD98059 (38). This may be clinically relevant since NO inhibits the activation of caspases and prevents the development of congestive heart failure (39). Estrogen also activates extracellular-regulated kinases 1 and 2 (ERK1/2) in cardiomyocytes (38), colon cancer (40), breast cancer (41), and bone (42,43), and inhibits ERK1/2 in VSMCs (44) and lung myofibroblasts (45). In the heart, ERα also selectively activates the 38-kDa isoform of MAPK (p38) to modulate the development of pressure-overload hypertrophy (13,14,46,47), which is consistent with recruitment of p38 in other models of cardiac hypertrophy (48,49). In endothelial cells, estrogen prevents disruption of the actin cytoskeleton during ischemia, prevents cell death, and enhances injury-dependent angiogenesis by rapidly and selectively activating the anti-apoptotic β isoform of p38 (p38β) and inhibiting pro-apoptotic p38α, leading to the increased expression of MAPK-activated protein kinase-2 (MAPKAP-2) kinase and phosphorylation of HSP27 (50). Downstream effects include preservation of stress fiber formation and membrane integrity, prevention of hypoxia-induced apoptosis, and induction of both endothelial cell (EC) migration and the formation of primitive capillary tubes (50).

It is possible that ERα might direct the activation of more receptor-proximal signaling complexes located at the plasma membrane. When overexpressed in cells, ligand-bound ERα induces the rapid phosphorylation of IGF1R and the activation of ERK1/2. Because these receptors co-immunoprecipitate in a ligand-dependent manner, a direct physical interaction between ERα and IGF1R could conceivably mediate the activation of ERK1/2 (51). In breast cancer cell lines, ligand-bound ERα promotes the rapid phosphorylation of the proteins Src and Shc, resulting in the formation of a Shc-Grb2- (growth factor receptor binding protein 2)- Sos (son of sevenless) complex (52), leading to downstream activation of Ras, Raf, and MAPK. Similarly, in both breast cancer and prostate cancer cells, E2 treatment induces the association of ERα phospho-Tyr[537] with the Src SH2 (Src homology 2) domain, leading to activation of the Src-Ras-ERK pathway and cell cycle progression (53,54). Additionally, in breast cancer cells, Src modulates PI3K-Akt signaling through a reversible cross-talk mechanism whereby the ligand-bound ER forms a ternary complex composed of ERα, PI3K, and Src (55). Cross-talk between PI3K and Src has also been observed in osteoclasts and bone marrow cells (56, 57).

Non-nuclear signaling can also amplify the nuclear, transcriptional activity of ERα. For example, in lactotroph cells, E2 rapidly activates ERK1/2, leading to increased transcription of the prolactin (*PRL)* gene, thus creating an additive effect on *PRL* expression by complementing the direct ERE-dependent transcriptional activation of *PRL* by ERα (58). Non-nuclear ERα activity can also elicit ERE-independent transcriptional activation. In cardiac myocytes, E2 rapidly increases ERK1/2-dependent expression of the early growth response-1 gene (*egr-1*) by inducing the recruitment of serum response factor (SRF) to serum response elements (SREs) in the *egr-1* promoter (59).

Growth factors such as EGF and IGF-1 can stimulate the nuclear activity of ERα through a non-nuclear, E2-independent mechanism. Through the cross-talk of molecular networks, mitogenic extracellular signals are translated into cell cycle progression or, in cancer cells, into hormone-independent proliferation (60). EGF- and IGF-1–mediated stimulation of MAP kinases result in the direct phosphorylation of ERα on Ser[118] (42,61,62), which enhances the binding of p68 RNA helicase (63), and promotes AF-1-dependent transcriptional activity in uterine (64,65) and ovarian adenocarcinoma cells (66). Nuclear coregulator proteins can also be phosphorylated by ERK1/2 leading to increased transcriptional activity (67). Lastly, Src may enhance AF-1 function of ERα through either a Src, Raf-1, mitogen-activated ERK kinase (MEK) and ERK pathway that leads to phosphorylation of Ser[118], or a pathway that includes Src, MEK kinase (MEKK), Jun N-terminal kinase (JNK) kinase (JNKK), and JNK, and that regulates AF-1-associated coactivators (68).

MECHANISMS FOR ERα ACTIVITY AT THE PLASMA MEMBRANE

Membrane binding sites for E2 were first implicated in 1977 (26), and additional indirect evidence for a membrane-associated ERα comes from immunohistochemistry (69,70), overexpressed nuclear receptors (71), or studies with membrane-impermeable ligands (72–74). The trafficking of ERα to different cellular compartments may be regulated by the nature of the stimulation; for example, in VSMCs transfected with ERα, MAPK activation mediates the nuclear translocation of ERα from the membrane fraction by both E2-dependent and -independent mechanisms (75). However, because ERα has no intrinsic kinase or phosphatase activity, does not have hydrophobic stretches that could represent transmembrane domains, and lacks myristoylation and palmitoylation sequences that could anchor it to the membrane, membrane localization of the receptor seems unlikely. Alternatively, the receptor may associate with membrane caveolae: in fractionated plasma membranes from endothelial cells (ECs), ERα is localized to caveolae, and E2 stimulates eNOS in isolated caveolae in an ERα- and calcium-dependent manner (76–78). There is evidence that within the caveolae of ECs, HSP90, eNOS, and cav-1 (caveolin-1, the coat protein of caveolae) exist in a heterotrimeric complex that modulates eNOS activity depending on intracellular calcium levels (79,80).

Non-nuclear ERα signaling also involves membrane-associated heterotrimeric G proteins. In Chinese hamster ovary (CHO) cells transfected with ERα cDNA, treatment of membrane fractions with estrogen activated $G\alpha_q$ and $G\alpha_s$ and rapidly stimulated inositol phosphate production and adenylyl cyclase activity, respectively (71). G protein activation also occurs in ECs, where E2 activation of eNOS can be inhibited with the ER antagonist ICI 182,780, RGS-4 (a regulator of G protein signaling specific for $G\alpha_i$ and $G\alpha_q$), or pertussis toxin (specific for $G\alpha_i$) (81).

POSSIBLE NEW ESTROGEN RECEPTORS

Non-nuclear signaling may involve a receptor altogether distinct from the classical ERα. In macrophage cells, E2 and E2-BSA induce a rise in intracellular calcium that is inhibitable with pertussis toxin (82,83). The existence of an E2-GPCR in the hippocampus has also been hypothesized, where E2 stimulation potentiates kainate-induced currents through modulation of PKA activity (84).

The most notable evidence that estrogen's non-nuclear effects are mediated by a receptor distinct from ERα or ERβ has come from studies in the cerebral cortex, where estrogen rapidly stimulates tyrosine phosphorylation of Src, leading to subsequent Shc–Grb2 complex formation upstream of ERK and B-Raf activation (85,86). The pathway is not inhibitable by ICI 182,780 in cortical explants from ERα-deficient mice, suggesting that a new receptor, responsive to E2 but insensitive to ICI 182,780, mediates non-nuclear neuronal differentiation.

The nature of the ERα that mediates the non-nuclear effects of estrogen clearly requires further definition: the distinction between classical and atypical ERα might be made using cells cultured from complete ERα knockout mice, and ERα-truncated mutants might provide insight into the specific domains that mediate non-nuclear effects.

NON-NUCLEAR PHARMACOLOGICAL TARGETS

Nonetheless, an increasingly detailed understanding of the ER signaling network and its pleiotropic cellular effects have made the receptor an attractive pharmacological target. Selective estrogen receptor modulators (SERMs) are ER ligands which can have varying degrees of agonist or antagonist activities depending on the cell, promoter and coregulator context (87,88) (Table 1).

Tamoxifen, the prototypical SERM, renders indirect cardiovascular protective effects by reducing the amounts of serum total cholesterol and low-density lipoprotein (LDL) (89). Unfortunately, its strong agonist activity in the endometrium leads to endometrial hyperplasia and low-grade cancers. Raloxifene, a non-steroidal compound, is similar to tamoxifen but it is less agonistic in the endometrium (90). Though administered primarily for bone preservation, raloxifene also reduces serum triglycerides and serum fibrinogen levels (91). Like estrogen, raloxifene and its analog LY117018 (92) stimulate eNOS activity in endothelial cells through PI3K- and ERK-dependent pathways, respectively (93), both of which may be involved in coronary artery relaxation (94). Raloxifene also improves endothelium-dependent vasorelaxation in hypertensive rats by enhancing the expression and activity of NOS (95).

EM-800, a non-steroidal compound, has higher affinity for ERα than any other SERM (96). In addition to demonstrating potent antitumor activity in the uterus and breast, EM-800 may also prevent bone loss and lower serum cholesterol and triglyceride levels (97). Furthermore, in vitro studies in endothelial cells suggest that EM-800, like E2, enhances NO release by sequential activation of MAPKs and PI3K-Akt, implicating a direct vascular protective effect (98).

The tissue specificity of SERMs suggests context-specific regulatory mechanisms that depend on the ligand, the promoter of the target gene, and the combination and exchange of co-regulators (99,100). Breast cancer and pituitary lactotroph tumors, for example, demonstrate enhanced apoptosis and tumor shrinkage when transfected with adenovirus constructs containing dominant-negative ERα mutants (101). Because dominant-negative ERα and antiestrogens both recruit transcriptionally repressive proteins to the ERα DNA-binding complex (102,103), the co-regulatory proteins that govern ERα activity, in addition to the receptor itself, represent promising therapeutic targets.

SUMMARY

We are just beginning to appreciate the complexity of ERα signaling. Future research efforts will undoubtedly reveal the intricacies of expression and translocation of endogenous ERα and possibly the identity of a new receptor that binds E2 and activates non-nuclear signaling. Furthermore, the activity of coregulators and their role in distinguishing the nuclear and non-nuclear activities of ERα remain to be defined. A full understanding of these highly cell- and promoter-specific mechanisms will allow the development of specific agonists and antagonists that selectively elicit only the beneficial effects of estrogen.

Acknowledgements

Biography

James K. Liao, MD, (left) is the Director of the Vascular Medicine Research Unit at the Brigham and Women's Hospital, and is a Associate Professor of Medicine at Harvard Medical School. Address comments to JKL. E-mail jliao@rics.bwh.harvard.edu. Fax 617-768-8425

Karen J. Ho, MD, (right) is a member of the Vascular Medicine Research Unit, Brigham and Women's Hospital and Harvard Medical School.

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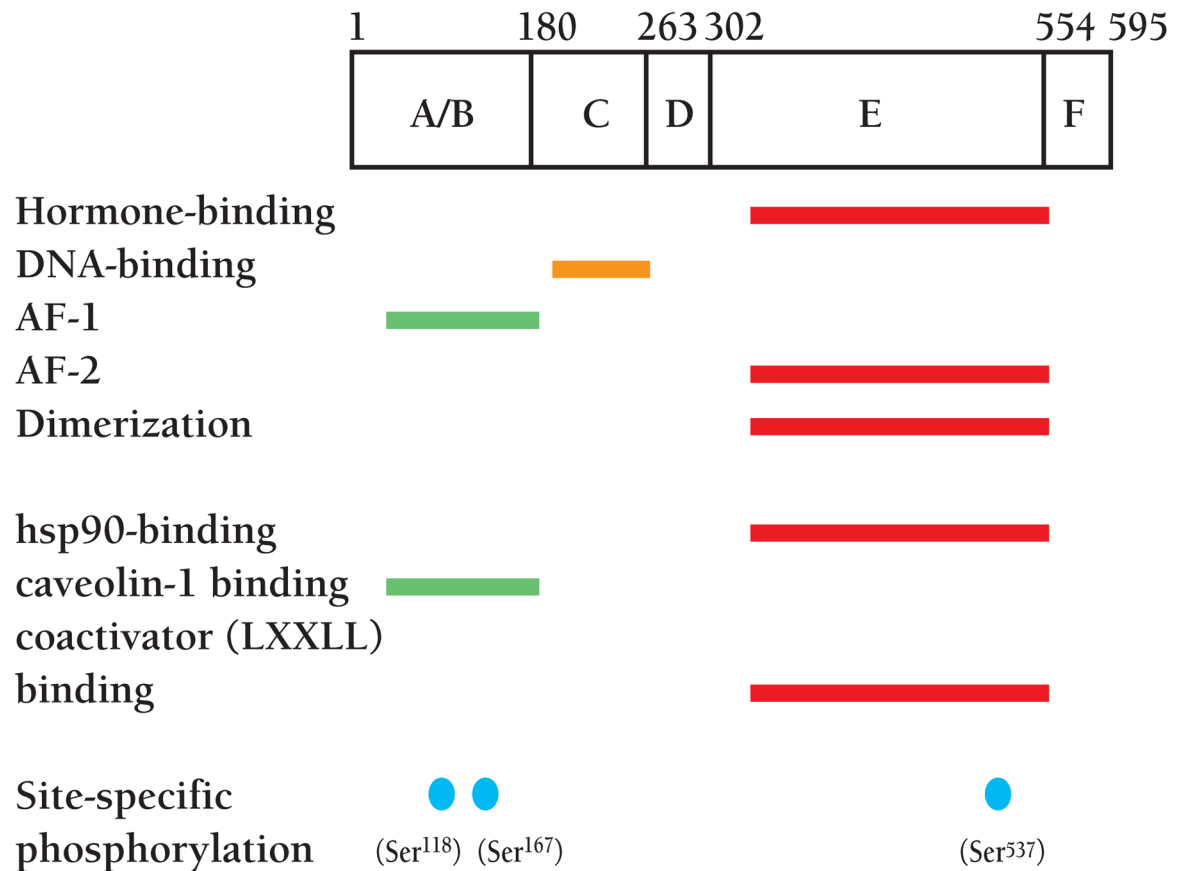
Figure 1.
Functional regions of the human estrogen receptor α (ERα). These domains include a ligand-independent transactivation function domain (AF-1), DNA-binding domain, hormone-binding domain and ligand-dependent transactivation function domain (AF-2). Putative regions of interaction with other proteins and sites of phosphorylation by various kinases are also shown.
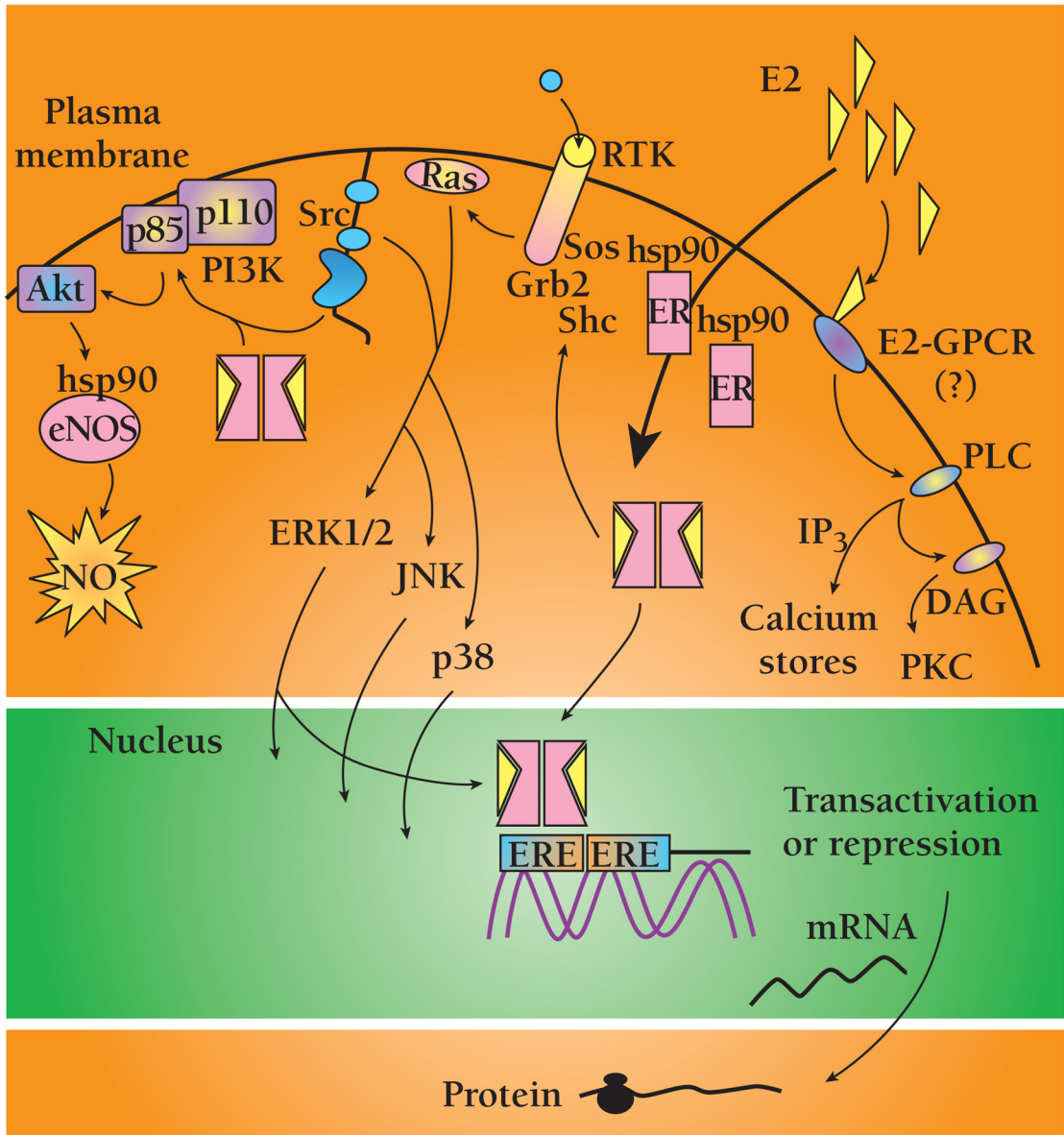
Figure 2.
Selected nuclear and non-nuclear activities of ERα. Details are described in the text.
Abbreviations: endothelial nitric oxide synthase (eNOS), nitric oxide (NO), heat shock protein
90 (HSP90), phosphatidylinositol-3′ kinase (PI3K), son of sevenless (Sos), growth factor
receptor binding protein 2 (Grb2), G protein coupled receptor (GPCR), protein kinase A (PKA),
protein kinase C (PKC), extracellular-regulated kinases 1 and 2 (ERK1/2), Jun N-terminal
kinase (JNK), 38-kDa isoform of MAPK (p38), estrogen response element (ERE).

TABLE 1

TISSUE-SPECIFIC EFFECTS OF SELECTED SERMS

| | Breast | Uterus | Vasculature | |
			Indirect effect	Direct effect
Tamoxifen	−	+	Lowers total cholesterol, no effect on HDL	?
				?
GW5638	−	−	Lowers total cholesterol	?
EM-800	−	−	Lowers total cholesterol and triglycerides	NO-mediated vasodilation
Raloxifene	−	−	Lowers total cholesterol and triglycerides, no effect on HDL	NO-mediated vasodilation
				ROS reduction
LY117018	?	−	Lowers total cholesterol	NO-mediated vasodilation