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The Expanding Complexity of Estrogen Receptor Signaling in the Cardiovascular System

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Abstract

Estrogen has important effects on cardiovascular function including regulation of vascular function, blood pressure, endothelial relaxation, the development of hypertrophy and cardioprotection. However, the mechanisms by which estrogen mediates these effects are still poorly understood. As detailed in this review, estrogen can regulate transcription by binding to two nuclear receptors, ER α and ER β , which differentially regulate gene transcription. ER α and ER β regulation of gene transcription is further modulated by tissue specific co-activators and co-repressors. Estrogen can bind to ER α and ER β localized at the plasma membrane as well as GPER to initiate membrane delimited signaling, which enhances kinase signaling pathways that can have acute and long term effects. The kinase signaling pathways can also mediate transcriptional changes, and can synergize with the estrogen receptor to regulate cell function. This review will summarize the beneficial effects of estrogen in protecting the cardiovascular system through ER-dependent mechanisms with an emphasis on the role of the recently described ER-membrane signaling mechanisms.

Keywords

Estrogen; signaling pathways; PI3 kinase

INTRODUCTION

Sex differences in cardiovascular disease are well documented. Premenopausal women have reduced cardiovascular disease and the occurrence increases after menopause^{1, 2}. Furthermore, many studies in animal models have reported less hypertrophy and less ischemia-reperfusion (I/R) injury in females compared to males^{3, 4}. Additional studies with ovariectomized females have also shown that addition of estrogen reduces hypertrophy and I/R injury⁵ (Figure 1 summarize the cardiovascular effects associated with estrogen). Large randomized clinical trials did not show a benefit with hormone replacement therapy (HRT)^{6, 7}. However, a recently update of the woman health initiative (WHI) examined different age groups and concluded that HRT “has a harmful effect on coronary heart disease

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DISCLOSURES

None.

among older women, whereas the results in younger women remain inconclusive”⁸. The data in this study (Manson et al.⁸, Appendix eFigure 3) report that in early menopausal women (age 50–59), conjugated equine estrogen (CEE) treatment alone was protective for myocardial infarction (HR 0.55, 95% CI 0.31–1.00), coronary artery bypass grafting/percutaneous coronary intervention (CABG/PCI) (HR 0.56, 95% CI 0.35–0.88), and all cardiovascular events (HR 0.84, 95% CI 0.66–1.06). There was a detrimental effect on deep vein thrombosis (HR 1.66, 95% CI 0.75–3.67). Interestingly the slight beneficial effects on myocardial infarction and CABG/PCI were reversed in older menopausal women, consistent with an age dependence. Although these data do not support the use of longer term HRT for reducing coronary heart disease, the data are consistent with the hypothesis that the beneficial effects of estrogen are reduced and ultimately reversed with age. The mechanisms responsible for the loss of protection with age are unclear, but it demonstrates that we do not fully understand the protective mechanisms in females and the role of estrogen. Although it is recognized that sex differences are due to many factors independent of estrogen, it is clear that estrogen has major effects in mediating sex differences in the cardiovascular system. This review will focus on estrogen signaling mechanisms in the cardiovascular system. In addition to the well-established action of the nuclear estrogen receptor-estrogen complex to modulate gene transcription, estrogen can also bind to several different estrogen receptors (ERs) located at the plasma membrane and activate membrane delimited signaling. The cross-talk between these signaling mechanisms leads to complex downstream signaling. In this review we will focus on estrogen signaling with an emphasis on the role of the more recently described membrane signaling mechanisms.

ER signaling has increased in complexity over the last 2 decades. Prior to 1996, only one ER was identified, which is now referred to as ER α , but at the time was simply called the ER. All the effects of estrogen were attributed to its binding to this one receptor which acted as a ligand regulated transcription factor. The complexity of estrogen signaling has grown over the last 20 years. There are now 3 ERs (ER α , ER β and an orphan G-protein coupled receptor, GPR30 now known as GPER) as well as several splice variants, and ER signaling occurs via the traditional regulation of transcription as well as by activating membrane signaling cascades.

The need for a better understanding of the mechanisms of estrogen signaling is clearly emphasized by the recent findings suggesting that there are sex differences in response to cardiovascular drug treatment and the suggestion that the failure of some small clinical trials could be related, at least in part, to sex differences in response to the drug^{9, 10}. In fact, the NIH has recently called for the inclusion of both sexes in preclinical trials¹¹. Without a better understanding of the complexity of estrogen signaling in the heart, it will be difficult to unravel the mechanisms by which estrogen might be involved in protection in pre-menopausal females and why it fails to protect in late post-menopausal women. This lack of understanding can also complicate the design and interpretation of clinical trials.

1. Estrogen initiates acute and chronic effects by binding to estrogen receptors

The physiological effects of estrogen are mediated by estrogen binding to ERs. With classical ER signaling, estrogen binding to ER α or ER β initiates translocation to the nucleus where the ER-estrogen complex acts as a ligand gated transcription factor initiating changes in gene expression. However estrogen can also bind receptors localized at the plasma membrane, leading to activation of acute signaling pathways often referred to as “non-genomic” or membrane delimited signaling. However, the term non-genomic is somewhat misleading because activation of acute signaling pathways at the membrane can also indirectly lead to changes in gene expression. Recent studies have demonstrated that post-translational modifications of ER α and ER β can influence their cell localization and activity^{12–14}. In addition to ER α and ER β , GPER has been shown to localize to membranes and activate acute signaling pathways following estrogen binding.

2. Genomic estrogen action

ER α and ER β are localized in both the cytosol and the nucleus and upon binding estrogen they can translocate to the nucleus, bind to DNA and modulate gene expression. ER α and ER β are highly homologous; they share about 95% homology in their DNA binding domains, but their ligand-binding domains share only 56% of amino acid sequence identity¹⁵. Estrogen-activated ERs alter gene expression by (i) directly binding to DNA, (ii) indirectly binding DNA through other transcription factors or (iii) ligand-independent binding (Fig 2). Estrogen binding to ER α promotes receptor dimerization and facilitates receptor binding to the consensus estrogen response element (ERE) in the DNA sequence, thus mediating its genomic effects. Co-activators and co-repressors are recruited to activate or inhibit gene expression¹⁶ (Fig 2, Panel A). Estrogen can also alter gene expression via non-ERE regulatory mechanisms. ERs can tether to activator protein-1 (AP1) and specificity protein 1 (Sp1) regulating gene expressions. Burns et al¹⁷ showed that mutation of a region of ER inhibits binding between ERs and transcription factors and blocks this signaling. Also consistent with the concept that ER binding via transcription factors is independent of EREs, Jakacka and colleagues reported that an ERE is not necessary for activation and inactivation of AP1-dependent transcription¹⁸ (Fig 2, Panel B). Interestingly, activation of kinase signaling pathways by growth factors or estrogen activation of membrane ERs can lead to phosphorylation of ER and trigger its binding to DNA, thereby modulating of transcription. A specific serine phosphorylation site on ER has been shown to be important for ligand-independent activation of transcription¹⁹ or for binding to ERE (Fig 2, Panel C and D). This mechanism was elucidated when it was observed that growth factor activation of uterine proliferation was lost in mice that lacked ER α ²⁰. Prior to these studies it was not appreciated that ER was required for growth factor dependent proliferation of the uterus²⁰. These results led to the concept that phosphorylation of ER can lead to its ligand independent effects on transcription.

Estrogen has been shown to regulate the expression of a large number of cardiac genes^{21–34}. Overall, different genes are found to be regulated in different studies and it is difficult to

draw general conclusions. Gene regulation depends on the context of the cell, thus the estrogen dependent changes in transcription might vary with age, sex, disease and tissue. To address some of these issues, Devanathan et al developed a mouse model of cardiomyocyte-specific deletion of ER α to characterize the role of ER α in the heart independent of systemic effects. Microarray data revealed differential variations in the expression of 208 genes compared to wild-type heart³⁵. A genome-wide expression profiling of estrogen-treated cardiomyocyte from men and women showed that 36 estrogen-dependent genes are regulated in a sex-specific manner confirming that estrogen effects are tissue- and sex-specific³⁶.

ER α versus ER β

To further complicate the analysis of ER regulated genes, ER α and ER β differentially regulate gene expression, and there can be tissue and temporal variations in the protein levels of ER α and ER β ^{37, 38}. Also, expression of co-activators and co-repressors can differ^{37, 39–41} and the recruitment of co-regulators depends in part on the ligand bound. For example, tamoxifen recruits co-activators in endometrium, but it is an antagonist in breast recruiting co-repressors⁴². See Hall et al for a more detailed review on genomic estrogen receptor signaling¹⁶ and Rosenfeld et al for a discussion of the roles of coactivators and corepressors in ER signaling⁴³. In mouse aorta, O'Lone et al reported that ER α and ER β regulate a distinct set of genes⁴⁰. They reported that estrogen activation of ER α primarily results in up-regulated gene expression, whereas ER β primarily enhances down-regulation of gene expression. In contrast to the study of O'Lone, a gene array study in ovariectomized female mice showed that perfusion of the heart for 2h with an ER β selective agonist primarily led to up-regulation of gene expression with only a few genes down-regulated⁴¹. These conflicting results might be due to differences in aorta versus ventricular myocytes (e.g. differences in levels of co-repressors and co-activators) or because of different durations of estrogen treatment; as mentioned estrogen can act in a time- and tissue-dependent manner⁴⁴. For example, in endothelial cells ER α upregulates endothelial nitric oxide synthase (eNOS), while in cardiac muscle ER β mediates up-regulation of eNOS^{45, 46}. Also, studies in vascular smooth muscle cells showed that inducible NOS (iNOS) expression is enhanced by ER β and repressed by ER α ⁴⁷. Furthermore, ER β blocks iNOS gene up-regulation in endothelial cells⁴⁸. ER α and ER β can also synergize to regulate protein and enzyme activity. For example, in vascular endothelial cells, ER α regulates activation of superoxide dismutase 2 (SOD2), whereas ER β regulates SOD2 basal expression. SOD2 up-regulation results in a decrease in reactive oxygen species (ROS) generation with lower mitochondrial dysfunction and vascular damage⁴⁹. Table 1 summarizes the known functions associated with ER α and ER β gene regulations.

Overall, estrogen regulation of gene expression is complex; ER α up-regulates some genes, while others are unaffected or down-regulated by ER β and vice versa. Also, the relative levels of ER α and ER β and the co-repressors and co-activators can change with sex, age, disease, and tissue thereby altering the response to estrogen. Additional studies are needed to better characterize the factors that contribute to tissue specific ER regulation of gene expression in the cardiovascular system. Furthermore, although there are a number of studies examining ER regulation of gene expression, very few studies have examined sex

differences or estrogen mediated differences in protein expression^{50–52}. There are a number of studies showing that changes in gene expression do not always translate to changes in protein expression, and therefore it is important to characterize the effects of sex and estrogen at the protein level. In addition to regulating mRNA transcription, estrogen is likely to regulate RNA processing, splicing, mRNA and protein stability, and protein post-translational modifications^{53–57}. We have just begun to understand these sex differences in protein levels and additional studies are needed.

3. Membrane delimited (Non-genomic) signaling

A rapid response to estrogen was first reported in the 1960s. Szego et al showed that estrogen increased the cAMP concentration within minutes, paving the way for recent studies on the non-genomic response of estrogen⁵⁸. It is established that physiological concentrations of estrogen can have rapid effects, which occur independent of protein synthesis or gene activation, and it is generally accepted that these acute effects of estrogen are transmitted by signaling pathways through activation of ERs localized at the plasma membrane^{59, 60}. In addition to the plasma membrane, ERs have also been localized to organelles including mitochondria⁶¹ and endoplasmic reticulum^{30, 62, 63}; however the effects of ERs in mitochondria and endoplasmic reticulum are poorly understood and will not be discussed here.

Investigations of non-genomic estrogen action and its contribution to cellular function have been facilitated by the development of an estrogen conjugated to bovine serum albumin (BSA). However, the cross-link between estrogen and BSA was through a key binding site for ERs making the experiments problematic as the conjugation inhibited the physiological effects of estrogen binding^{64–66}. Recently, Katzenellenbogen and coworkers developed an estrogen-dendrimer conjugate (EDC), an estrogen-macromolecule conjugate in which estrogen is attached to a large and positively charged polyamidoamine dendrimer⁶⁷. EDC provides a new tool to investigate the non-nuclear signaling mechanisms of estrogen.

3.1 ER α and ER β – membrane delimited

3.1.1 ER α and ER β both localize to the plasma membrane—It is estimated that approximately 5–10% of total ERs are localized to the plasma membrane⁶⁸. The relative amount of ER α and ER β at the plasma membrane can differ among cell types; for example in breast cancer cells ER α is found to be less than ER β ⁶⁹, meanwhile in reproductive tissue ER α is more abundant than ER β ⁷⁰. ERs distribution is poorly studied in cardiovascular tissue, however both the ER α and ER β are found to be present at the plasma membrane of vascular endothelial cells (EC)⁷¹. ER α and ER β are tethered to the plasma membrane by a mechanism involving post translational modifications such as palmitoylation, which has been shown to increase association of the receptor with the plasma membrane^{30, 62}.

Several laboratories have conducted investigations to confirm the localization of ER α to the plasma membrane. Using three different ER α antibodies, Chambliss and colleagues found ER α in EC plasma membranes, and they further demonstrated the presence of the ER α protein in a plasma membrane fraction isolated from the cell. They were also able to detect ER α in the caveolae using subfractions of caveolae and non caveolae⁶². The same group

later reported that ER α and eNOS co-localized in the caveolae. This group also found that ER β is present in caveolae from EC and has a non-genomic signaling response, through eNOS stimulation⁷². Chambliss et al. further confirmed that the same isoform of ER α (or ER β) was present in purified plasma membranes and in the nucleus³⁰. Finally, Pedram et al confirmed in human breast cancer cells, using a mass spectrometry approach, that membrane and nuclear ERs are the same protein⁷³.

Several sites have been identified on ER α , which are involved in its localization to the plasma membrane. The interaction between ER α and caveolin-1 requires Ser-522, located in the ligand-binding domain, which has been reported to facilitate the binding of ER α to caveolae^{12, 62}. Also, palmitoylation of Cys447 on the ligand-binding domain of ER α has been demonstrated to be crucial for ER α localization to the membrane^{12, 74}. Finally immunoprecipitation studies demonstrated that amino acids 251–260 and 271–595 of ER α bind directly to G α i and G $\beta\gamma$ ⁷⁵. These modifications and their role in specific cell types will be discussed later.

The full-length ER α seems to be the predominant form associated with the plasma membrane and has been shown to initiate rapid actions of estrogen. However, an N-terminus-deleted splice variant, ER α 46, has been shown to co-localize with caveolin-1 in caveolae and it seems to have a reduced transcriptional activity compared to the full-length ER α ⁷⁶. ER α 46 has been found co-immunoprecipitated with eNOS⁷⁷ leading to eNOS activation and nitric oxide release⁷⁸.

3.1.2 Trafficking to the plasma membrane—As mentioned above, Ser522 is important for physical association of ER α with caveolin-1, and subsequent translocation to the plasma membrane¹². Indeed, one study showed that ER α localization at the plasma membrane is lost in caveolin-1 null cells, and ER α is only found in the nucleus¹². However, another study reported that the mutation of Ser522 in Chinese hamster ovary cells is not sufficient to block the ER α translocation to the plasma membrane, suggesting that Ser522 is not the only residue responsible for membrane association¹². Acconcia et al found that Cys447 of human ER α is a site for palmitoylation and this modification promotes the association of ER α with caveolin-1^{13, 74}. Mutation of this site blocked ER α translocation to the plasma membrane in cancer cell lines⁵³. In 2007, Levin's group identified a highly conserved amino acid motif in the estrogen binding domain of both human and mouse ER α and ER β that mediates membrane translocation via palmitoylation, and Cys447 is part of this motif¹⁴. Therefore, palmitoylation of Cys447 is essential for caveolin-1 interaction and membrane translocation. However, additional sequences flanking this cysteine residue are needed for optimal palmitoylation. Mutation of key residues of ER for palmitoylation, such as phenylalanine or tyrosine at position –2, significantly reduces membrane localization and non-genomic actions of ER α ¹⁴. Palmitoylation occurs only on ER monomers, therefore enhancing ER dimerization, such as with estrogen binding, limits the available receptors that can undergo palmitoylation and translocate to the membrane. Since the percentage ERs localized at the plasma membranes are only 5–10% of the total amount present in the cell, it is important to define the proteins and mechanisms that regulate the ERs trafficking to plasma membrane. Heat shock protein 27 (HSP27) was identified by Razandi et al as a promoter of ER palmitoylation⁷⁹. Using a proteomic approach, they found that HSP27 binds to the

palmitoylation site of ER α and promotes palmitoylation of ER α monomer. Another group found that ER α can localize to caveolae indirectly via the scaffold protein striatin, forming complexes with G α i and G α proteins⁸⁰.

3.1.3 ER α and ER β signaling—ER α was first shown to localize to the plasma membrane and to activate phosphatidylinositol-3-OH kinase (PI3K) signaling⁸¹. ER α and ER β have been shown to play important roles in modulating cardiovascular disease. As discussed above, ERs localized at the plasma membrane initiate rapid signal transduction through kinase cascades involving PI3K^{82, 83}, mitogen-activated protein kinase (MAPK)^{84, 85}, and endothelial nitric oxide synthase (eNOS) activation^{86, 87}. Rapid signaling by estrogen action was shown to lead to increased nitric oxide within minutes after addition of estrogen⁸⁸. Nitric oxide generation is involved in many beneficial effects on the cardiovascular system. Nitric oxide induces EC growth and migration, vasodilatation, and platelet inhibition, resulting in prevention of thrombus formation and leukocyte-EC adhesion^{71, 89}.

3.1.3.a Endothelial cells: Endothelial homeostasis is important in cardiovascular health and endothelial dysfunction is associated with an increased cardiovascular risk⁹⁰. The rapid activation of eNOS is an important mechanism in vascular relaxation, EC migration and proliferation. Initial studies showed that an estrogen-BSA conjugate stimulates eNOS phosphorylation in cultured EC⁶⁶. Estrogen can activate eNOS by multiple signaling pathways as illustrated in Figure 3A. Upon estrogen binding, membrane localized ER α forms a complex with p85 α , the regulatory subunit of PI3K⁸³ and with SH2 domain of c-Src mediated by the phosphorylated Tyr-537 of ER α ^{78, 91} and/or by methylation of Arg260 of ER α ⁹². The formation of the ER α /PI3K complex leads to activation of protein kinase B (Akt), extracellular-signal-regulated kinases 1/2 (ERK1/2), and phosphorylation and activation of eNOS. ER α can also activate MAPK signaling and stimulate nitric oxide production by eNOS⁸⁷. Indeed, mice treated with estrogen showed increased eNOS activity and decreased vascular leukocyte accumulation after I/R injury and this vascular protective effect of estrogen was abolished in the presence of inhibitors of PI3K or eNOS⁸³. ER α can also interact with the G proteins G α i and G β γ through amino acids 251–260, and this interaction activates eNOS and monocyte adhesion, as well as stimulating cell migration⁷⁵. A single alanine substitution generated within the G α i binding domain of ER α blocked the ability of estrogen to induce ERK1/2 and eNOS activation. Also, blocking formation of the ER α -G α i complex prevented EC cell migration⁹³. Recently, Chambliss et al used EDC to demonstrate that EC proliferation and migration occurs via ER α -G α i-Src kinase complex formation and resultant eNOS activation⁹⁴. They also demonstrated that EDC can attenuate the development of neointimal hyperplasia following vascular injury. Interestingly, EDC did not cause endometrial carcinoma cell growth in vivo or increase cell proliferation in breast cancer cells. The activation of PI3K upon ER α -G α i complex formation increases the activity and expression of the metalloproteinase (MMP) MMP2 and MMP9 leading to EGF receptor stimulation, which activates the MAPK kinase signaling pathway⁸⁴.

Immunoprecipitation experiments demonstrated that ER α can bind the adaptor striatin through amino acids 183–253 of ER α . Estrogen promotes the formation of a complex

containing ER α , striatin and G α i leading to activation of ERK1/2, Akt and eNOS pathways stimulating EC migration^{80, 95}. Beside nitric oxide generation, the non-genomic ER signaling pathway also modulates intracellular calcium homeostasis at physiological estrogen concentration (10⁻⁹M) in human EC, causing a rapid increase in intracellular calcium⁹⁶. Treatment with estrogen conjugated to BSA caused a rise in calcium concentration in rat EC whereas treatment with the ER antagonist ICI182,780 blocked this effect⁹⁷. Taken together, estrogen activation of membrane ERs leads to rapid signaling mechanisms that target nitric oxide signaling, thus modulating EC proliferation, migration and vascular relaxation.

3.1.3.b Vascular smooth muscle cells: The proliferation of vascular smooth muscle cells (VSMC) plays a crucial role in vascular diseases, especially in atherosclerosis. Similar to EC, ER α and ER β are associated with caveolin-1 at the plasma membrane of VSMCs. Several studies demonstrate that estrogen rapidly (within 30 minutes) inhibits VSMC proliferation^{98, 99}. Upon estrogen treatment, ER α stimulates the activity and expression of several phosphatases, such as MAP kinase phosphatase-1 (MKP-1), Src homology region 2 domain-containing phosphatase-1 (SHP-1), phosphatase and tensin homolog (PTEN), and protein phosphatase 2A (PP2A). Activation of these phosphatases, which reverses phosphorylation, result in a decrease of cell growth and migration⁹⁹⁻¹⁰¹ (Fig 3, Panel B). Consistent with these findings, a recent study showed that estrogen significantly blocks VSMC proliferation, and decreases Akt phosphorylation due to enhanced PP2A activation mediated by a complex formed between ER α and PP2A¹⁰². They also used VSMC derived from a transgenic mouse line overexpressing a peptide that prevents ER trafficking to the plasma membrane and demonstrated that in this transgenic mouse, cell proliferation was not inhibited upon addition of estrogen. Similar results were obtained by other groups using transgenic mice overexpressing a peptide that prevents ER α from interacting with striatin, a scaffold protein essential for ER binding to the caveolae. These studies demonstrated that the ability of estrogen to stimulate VSMC growth after carotid artery injury was lost in mice overexpressing the disrupting peptide⁹⁵. Microarray analysis of ex vivo aorta from these mice showed that the transcriptional response was greatly altered by blocking the non-genomic signaling. The genes showing different responses in the transgenic versus wild type mice were associated with vascular function, indicating that acute estrogen signaling also plays a key role in physiological vascular gene regulatory responses. Further studies are needed to better understand the molecular mechanisms of non-genomic signaling as well as the effects on gene transcription. A better understanding of these mechanisms could contribute to the development of new treatments to improve vascular health.

3.1.3.c Cardiomyocytes: There are very few studies examining the non-genomic role of ER α and ER β in cardiomyocytes. No studies have been done to distinguish genomic and non-genomic actions using transgenic models or extranuclear ER agonists. Estrogen was shown to have a role in cardioprotection against I/R injury. Short-term estrogen treatment (15 minutes before ischemia and 5 minutes at the beginning of reperfusion) improved heart function similar to that seen with pacing postconditioning^{3, 103}. In addition, these beneficial effects of estrogen were not observed in the presence of a NOS inhibitor, suggesting that nitric oxide production via ER activation plays a key role in this process¹⁰³. Also, there are a

number of studies showing that estrogen prevents cardiac hypertrophy; in particular through ER β ^{4, 104}. Levin's group demonstrated that estrogen is not able to prevent angiotensin-induced hypertrophy and fibrosis in ER β knock out mice¹⁰⁵. They provide data showing that rapid estrogen signaling acts on cardiac hypertrophy sequestering the transcription factors in the cytoplasm of cardiomyocytes to prevent target gene activation. Indeed, estrogen through ER β can activate the modulatory calcineurin-interacting protein 1 gene blocking calcineurin activation in myocytes²². Histone deacetylase (HDACs) are important in regulation of cardiac hypertrophy: class II is involved in signaling that inhibit this disorder, whereas class I promotes hypertrophy¹⁰⁶. Recently, Pedram et al demonstrated that angiotensin regulates gene and protein level of both class I and II HDACs and rapid estrogen action through ER β blocks these angiotensin actions¹⁰⁷. Estrogen causes inhibition of calcium flux through a G α i-dependent mechanism, inhibiting Ca²⁺/calmodulin-dependent protein kinase II-induced class II HDAC4 phosphorylation and nuclear translocation. Also, angiotensin stimulates protein kinase C and D with consequent phosphorylation of class II HDAC5 and estrogen is able to prevent these effects¹⁰⁷. In summary, these findings suggest an important role for estrogen in cardioprotection due to rapid estrogen action. Additional studies should be conducted using the novel extranuclear ER agonists together with ER null transgenic mice to better understand the mechanisms involved in the protection against heart disease.

3.2 G-protein coupled receptor signaling

3.2.1 G-protein coupled receptor localization—It has recently been shown that estrogen can signal through an orphan G-protein-coupled receptor, GPR30^{63, 108–110}, also known as G protein-coupled estrogen receptor 1 (GPER). Two independent groups found that estrogen can directly bind to GPER at the membrane in monkey kidney fibroblast cells and breast cancer cells^{63, 109}. Haas and colleagues later discovered GPER expression in VSMC of human arteries and veins and confirmed that this novel receptor is regulated by estrogen¹¹¹. There is some debate regarding the cellular location of GPER. Prossnitz's group expressed GFP-GPER in monkey kidney fibroblasts and images obtained using confocal fluorescence microscopy revealed that GPER is localized to the endoplasmic reticulum and the Golgi apparatus, but not to the plasma membrane⁶³. They also found that estrogen binding to GPER increases calcium mobilization by a signaling pathway involving epidermal growth factor receptor transactivation¹⁰⁸ rather than the more common mechanism involving ER activation of phospholipase C (PLC)–dependent inositol 1,4,5-trisphosphate production. In contrast, other groups reported that GPER is localized to the plasma membrane^{112, 113, 114}. A possible explanation for these discrepant data has been proposed by Filardo's group, which showed that GPER undergoes constitutive endocytosis resulting in a rapid (half life of 30 min) down-regulation. The recycled receptor that is present in the endosomes and endoplasmic reticulum levels might result from high levels of synthesis of the protein¹¹⁵. In summary, GPER localization appears to be heterogeneous indicating that GPER can localize both at the plasma membrane and in specific intracellular sites. However, whether GPER is functionally active in the endoplasmic reticulum membrane is debated.

3.2.1.a Vascular (EC and VSMC) Effects of GPER: Several studies investigated the role of GPER in vascular cells. Interestingly, the classical ER α and ER β antagonist, ICI182,780,

was not able to attenuate estrogen-induced vasorelaxation in either canine coronary or small rat arteries^{116–118}. Although ICI 182,780 is an antagonist of ER α and ER β , it is an agonist of GPER. Furthermore, removal of ER α and ER β did not abolish estrogen-dependent responses in the cardiovascular system¹¹⁹. These results indicated that estrogen was not acting solely through ER α or ER β , but that another receptor was involved. This led to the suggestion that activation of GPER might be responsible for the vasodilatory effects of estrogen. Several studies using the selective GPER agonist (G-1) were conducted confirming its ability to initiate vascular relaxation¹²⁰. Acute G-1 treatment induces relaxation on porcine coronary arteries¹²¹, on rat aorta¹²², and human internal mammary arteries¹²³. Infusion of G-1 to normotensive rats, results in an acute reduction in blood pressure. Furthermore, GPER knock out mice did not exhibit a vascular response on addition of estrogen¹²³. Moreover, in hypertensive mRen2.Lewis rats, G-1 treatment leads to improved cardiac relaxation and reduced myocyte hypertrophy¹²⁴. Furthermore, GPER agonist treatment induces vasodilation in female rats¹²⁵. It has been suggested that G-1 results in 30–40% vasorelaxation in vivo¹²⁶, however the mechanism mediating this effect is poorly understood. There are data reporting actions of GPER via endothelium-dependent and endothelium-independent mechanisms. Estrogen binding to GPER leads to activation of eNOS, thus increasing the production of nitric oxide in coronary ECs to relax these arteries¹¹⁶. Endothelium denudation or treatment with L-NAME, a NOS inhibitor, completely abolished the relaxation of porcine coronary arteries induced by G-1 action, consistent with a role for eNOS activation¹¹⁶. In contrast, several other studies demonstrated that G-1 treatment can still have an effect on vasorelaxation in endothelium-denuded aorta¹²² and porcine coronary arteries¹²⁷. It was also shown that inhibition of eNOS does not affect this vasodilation response. Yu et al suggested that the endothelium-independent effect of coronary smooth muscle was mediated by a large conductance calcium-activated potassium channel in porcine and human arteries¹²⁷. Moreover, treatment with an eNOS inhibitor did not block the G-1-induced relaxation, but relaxation was inhibited by blocking the potassium channel. A scheme of these mechanisms is shown in Figure 4.

The studies, suggesting that activation of GPER alone mediates vasodilation, contrast with studies suggesting that ER α and/or ER β active NOS and mediate vasodilation. The reason for this discrepancy is unclear, but it may be due to differences in models, specificity of agonists and antagonists¹²⁸ or cross-talk between different receptors.

Normally, VSMC proliferation is slow, however during stress, such as atherosclerosis or hypertension, these cells increase the rate of proliferation and migration¹²⁹. Estrogen decreases VSMC proliferation after injury.^{130–132} However, the anti-proliferative actions of estrogen do not require ER α or ER β as estrogen retains its anti-proliferative effect in ER α and ER β knock out mice, suggesting that other receptors such as GPER might play a role^{133, 134, 131}. Li and colleagues recently demonstrated in coronary VSMCs that estrogen activation of GPER can decrease cell proliferation and migration¹³⁵. Estrogen also has a role in regulating proliferation of vascular ECs, thus preventing excessive EC proliferation that can occur during atherosclerosis⁹⁴. Treatment with G-1 reduced DNA synthesis and endothelial proliferation by blocking the transition between G2 and M phases of the cell cycle in mouse microvascular ECs and also in human umbilical vein ECs¹³⁶, again showing a role for GPER.

3.2.1.b Cardiac effects of GPER: Ullrich et al have shown in cardiomyocytes that estrogen-dependent intracellular calcium signaling is independent of classic ERs, ER α and ER β ¹¹⁹. They treated cardiomyocytes from ER α and ER β knock out mice with serotonin and showed that the estrogen-mediated inhibition of Ca²⁺ influx and muscle contraction was not altered. Haas et al developed a GPER knock out mouse and confirmed that GPER stimulation is important for changing the intracellular Ca²⁺ concentration¹²³. Thus, estrogen may play a role through GPER by regulating the myofilament sensitivity to Ca²⁺ influx.

It has also been shown that estrogen-GPER activation protects against cardiac I/R injury¹³⁷. Deschamps et al demonstrated that acute treatment of perfused rat heart with G-1 is cardioprotective, as it improved the contractile dysfunction after ischemia. The protection afforded by G-1 was shown to be dependent on PI3K signaling as a PI3K inhibitor blocked G1-mediated cardiac protection¹³⁸. Also, GPER activation has been shown to inhibit mitochondria permeability transition pore opening after I/R injury, thereby protecting hearts from cell death¹³⁹. It was also reported that G-1 can reduce the inflammatory response in global ischemic injury¹⁴⁰. The discovery that GPER activation of acute signaling mechanisms results in myocardial protection provides the exciting possibility that GPER agonists can be used to initiate cardioprotection while avoiding the side effects of estrogen due to nuclear ER stimulation in the uterus and breast.

GPER studies in the cardiovascular field have been growing rapidly and overall these findings indicate a protective role of GPER in cardiovascular disease. Agonists of GPER are potential selective ER modulators (SERMs) and are likely to be good candidates for intervention in many cardiac dysfunctions. However, the molecular mechanisms downstream of GPER stimulation are still unclear and future research needs to be done to clarify the signaling pathways involved and to understand the potentially beneficial effects of this receptor.

4. ER membrane and genomic collaboration for regulation of gene expression

Although nuclear and acute effects of estrogen can individually lead to changes in cell signaling, gene expression and cell function, there is also cross-talk between the non-genomic and genomic pathways which work together to modulate cell and organelle function. Estrogen acts synergistically, coordinating the activation of kinase signaling and the genomic signaling to regulate gene transcription. Mice lacking the ER α palmitoylation site, in which signaling is only via nuclear DNA binding and not via membrane signaling, were generated and offer insight into the collaboration between non-nuclear and nuclear ER α and ER β signaling^{141,142}. Pedram et al found that nuclear ER α and ER β alone are not able to maintain normal reproductive organ development; these mice showed infertility, abnormal ovaries and pituitary hormone regulation¹⁴¹. Overall, they also demonstrated that loss of membrane-ER α compromises estrogen-induced expression of some important mRNAs in the uterus, mammary glands and ovaries. Moreover, Pedram et al developed a mouse that expresses the ligand binding domain of ER α that is targeted exclusively to the plasma membrane. After ovariectomy and treatment with an ER α agonist, they carried out

DNA microarray from liver mRNA. The majority of genes were regulated only by nuclear ER α , however mRNAs for cholesterol, triglyceride and fatty acid synthesis were comparably suppressed in membrane-only-ER α and wild type mice and completely absent in ER α knock out mice. Several transcription factors are regulated by protein kinase-mediated phosphorylation. These transcription factors might regulate gene expression by ERs directly binding to DNA (Fig. 2, Panel B). For example, AP-1 activity is regulated via MAPK-mediated phosphorylation¹⁴³. Estrogen can activate MAPK signaling that results in enhanced AP-1 DNA binding and lead to gene expression activation^{144, 145}. Also, activation of the PI3K-Akt signaling pathway by estrogen leads to nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) phosphorylation and activation resulting in enhanced expression of genes that contain an NF-kB binding site¹⁴⁶. Katzenellenbogen's lab recently reported that ~25% of estrogen-regulated genes are responsive with EDC treatment¹⁴⁷. Also, knockdown of ER α abolished gene expression due to EDC treatment. Conversely, the knockdown of GPER showed no effect¹⁴⁷, indicating that in this study the cross-talk between the non-genomic and genomic effects is mediated through ERs.

4. ER expression during aging

Premenopausal women have a lower incidence of cardiovascular disease compared with age-matched men. However, this advantage for women gradually disappears after menopause with the cessation of ovarian function and reductions in estrogen levels. Both the ER α and ER β have been localized in the cardiovascular system¹⁴⁸. The expression level of ERs appears to vary with gender^{38, 149, 150}. Whether ER isoforms are distributed differently in cardiovascular system and whether they change with aging or gender is an important area for further investigation and would partially explain the clinical conflicting evidence of estrogen treatment in cardiovascular disease. However, changes in ER expression and signaling pathway are poorly investigated. Estrogen has been shown to positively regulate ER levels¹⁵¹, and also other hormones such as progesterone and vitamin D negatively regulate ER levels¹⁵². Measured by western blotting ER α and ER β did not differ with age in aorta from spontaneously hypertensive rats¹⁵³. A study using immunofluorescence reported that vascular EC ER α expression in post-menopausal women is 33% lower than in the late follicular phase in premenopausal women^{153, 154}. In astrocytes, using immunohistochemistry and western blot analysis Arimoto et al reported an increase in ER α levels with aging¹⁵⁵. Using immunofluorescence Wu et al also found an increase in ER α with age in the hypothalamus¹⁵⁶. Alterations in methylation of CpG islands, a cytosine and guanine rich area in gene promoter regions, in the ER promoter have been reported to occur with age and disease and these changes could be involved in altering ER levels. Methylation of the ER promoter has been reported to increase with atherosclerosis¹⁵⁷, but to decrease in brain following stroke¹⁵⁸. In future studies it will be important to better define changes in expression and localization of ER with age, sex and disease in the cardiovascular system.

CONCLUSION

Although estrogen signaling has been studied for years in reproductive tissues, it is only in the past decade or so that its role in the cardiovascular system has been appreciated. It has become apparent that estrogen has effects on cardiovascular tissues. Estrogen has important

effects on lipid profile (such as lowering LDL cholesterol), vascular remodeling, blood pressure, endothelial relaxation, development of hypertrophy and cardioprotection. However, the mechanisms by which estrogen mediates these effects are still poorly understood. As detailed in this review, estrogen can regulate transcription by binding to two nuclear receptors, ER α and ER β , which differentially regulate gene transcription. These receptors are present at different ratios on different cells and their levels can change with time. ER α and ER β regulation of gene transcription is also modulated by tissue specific co-activators and co-repressors. Taken together estrogen can result in tissue- and temporal-specific regulation of gene transcription. Layered on top of tissue specific estrogen regulation of transcription, and adding to the complexity, is membrane delimited signaling. Estrogen can bind to ER α and ER β localized at the plasma membrane as well as GPER; estrogen binding enhances acute kinase signaling pathways that synergize with the estrogen receptor to mediated transcriptional changes. In addition, acute signaling cascades can lead to phosphorylation of estrogen receptors that can lead to ligand independent ER regulation of transcription (see Figure 2). Understanding these complexities of estrogen-ER signaling is necessary to better understand why premenopausal women have reduced cardiovascular disease but yet HRT was not beneficial, at least in older menopausal women. Furthermore a better understanding of what regulates ER α versus ER β and how the nuclear ER transcriptional activation synergizes with acute membrane ER signaling will allow better design of selective estrogen modulator which may provide beneficial effects of estrogen without unwanted side effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

Akt	protein kinase B
AP1	activator protein-1
CEE	conjugated equine estrogen
EC	vascular endothelial cells
EDC	estrogen-dendrimer conjugate
eNOS	endothelial nitric oxide synthase
ER	estrogen receptor
ERE	estrogen response element

ERK1/2	extracellular-signal-regulated kinases 1/2
GP_{ER}	G protein-coupled estrogen receptor 1
GPR30	orphan G- protein coupled receptor
HDACs	histone deacetylase
HRT	hormone replacement therapy
HSP27	heat shock protein 27
I/R	ischemia/reperfusion
iNOS	inducible nitric oxide synthase
MAPK	mitogen-activated protein kinase
MKP-1	MAP kinase phosphatase-
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
PI3K	phosphatidylinositol-3-OH kinase
PP2A	protein phosphatase 2A
PTEN	phosphatase and tensin homolog
SERMs	selective estrogen receptor modulators
SHP-1	Src homology region 2 domain-containing phosphatase-1
Sp1	specificity protein 1
VSMC	vascular smooth muscle cells
WHI	woman health initiative

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Figure 1. Effects of estrogen on the heart

Estrogen has many pleiotropic effects on the cardiovascular system. Estrogen can impact cardiovascular health and disease by direct effects: (i) on the vascular endothelial cells promoting vasorelaxation, cell proliferation and migration; (ii) on vascular smooth muscle cells decreasing cell proliferation and migration and (iii) on cardiomyocytes reducing LDL-cholesterol level and protecting against insulin resistance, infarct size and ischemia–reperfusion injury and cardiac hypertrophy.

Figure 2. Genomic ER signaling

A: Direct binding to DNA. Estrogen binding to ERs promotes receptor translocation to the nuclei. ERs bind to the consensus estrogen response element (ERE) in the DNA, mediating its genomic effects. Co-activators and co-repressors are recruited to activate or inhibit gene;
B: Indirectly binding DNA through other transcription factors. ERs can tether transcription factors (TF) such as API and Sp1 regulating gene expressions; **C & D:** Ligand-independent binding. ERs can be phosphorylated by kinases signaling (such as p38, ERK and Akt) activated by plasma membrane ERs signaling. Specific serine site phosphorylation of ERs can trigger its binding to DNA thus activating the transcription via ligand-independent binding or via ERE-binding.

Figure 3. Non genomic ER signaling

Panel A: Rapid ER signaling in vascular Endothelial Cells. ERs localize to caveolae by direct binding to caveolin-1 and scaffold protein striatin. Upon estrogen binding, ERs and the G protein form a complex leading to activation of tyrosine kinase Src, serine/threonine kinase PI3K (binding the subunit p38α), Akt, and MAPK. Kinases then directly activate eNOS by serine 1177 phosphorylation. The increased production of nitric oxide (NO) promotes endothelial cell vasodilatation, proliferation and migration. **Panel B:** Rapid ER signaling in vascular smooth muscle cells. ERs localize to caveolae by direct binding to

caveolin-1 and scaffold protein striatin. Upon estrogen binding, ERs activate several phosphatases leading to inhibition of kinases and finally resulting in a decrease of cell proliferation and migration.

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Figure 4. GPR30 activation via endothelium-independent or endothelium-dependent mechanisms

A: Endothelium-independent effect is mediated by a large conductance calcium-activated potassium channel leading to an increase in potassium efflux. This effect results in coronary artery relaxation. BK, Ca²⁺- and voltage-activated K⁺ channels. **B:** Endothelium-dependent mechanism. Estrogen binding to GPR30 leads to activation of eNOS raising the production of nitric oxide (NO) in coronary endothelial cells to relax these arteries.

Table 1Summary of known genomic function associated with ER α and ER β

ER α	ER β
<ul style="list-style-type: none"> Increases left ventricular mass and volume¹⁵⁹; Reductions of infarct size after myocardial infarction¹⁵⁹; Cardioprotection against ischemia-reperfusion injury^{160–162}; Regulates GLUT4 expression¹⁶³; Regulates cardiac growth¹⁶⁴. 	<ul style="list-style-type: none"> Reduces pathologic cardiac hypertrophy⁴; Prevents increases mortality in chronic heart failure^{165, 166}; Cardioprotection against ischemia-reperfusion injury⁴¹; Regulation of vascular function and blood pressure¹³³; modulates sex-specific response of the heart to exercise⁵⁰; Decreases inflammatory response⁴¹.