# Estrogen signaling in the cardiovascular system

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**Estrogen exerts complex biological effects through the two isoforms of estrogen receptors (ERs): ERα and ERβ. Whether through alteration of gene expression or rapid, plasma membrane-localized signaling to non-transcriptional actions, estrogen-activated ERs have significant implications in cardiovascular physiology. 17-β-estradiol (E2) generally has a protective property on the vasculature. Estrogen treatment is anti-atherogenic, protecting injured endothelial surfaces and lowering LDL oxidation in animal models. Increased NO production stimulated by E2 results in vasodilation of the coronary vascular bed, and involves rapid activation of phosphotidylinositol-3 kinase (PI3K)/Akt signaling to eNOS in carotid and femoral arteries. Both isoforms of ERs impact various vascular functions, modulating ion channel integrity, mitigating the response to arterial injury, inducing vasodilation, and preventing development of hypertension in animal models. In addition to reducing afterload by vasodilation, ERs have a direct antihypertrophic effect on the myocardium. E2-activated ERs (E2/ER) antagonize the hypertrophic pathway induced by vasoactive peptides such as angiotensin II by activating PI3K, subsequent MICIP gene expression, leading to the inhibition of calcineurin activity and the induction of hypertrophic genes. In models of ischemia-reperfusion, E2/ER is antiapoptotic for cardiomyocytes, exerting the protective actions via PI3K and p38 MAP kinases and suppressing the generation of reactive oxygen species. In sum, E2-activated ERs consistently and positively modulate multiple aspects of the cardiovascular system.**

Received December 30th, 2005; Accepted March 15th, 2006; Published July 7th, 2006 | **Abbreviations:** **AngII:** angiotensin II; **ANP:** atrial natriuretic peptide; **E2:** 17-β-estradiol; **eNOS:** endothelial nitric oxide synthase; **ERK:** extracellular signal-regulated kinase; **ERs:** estrogen receptors; **ET-1:** endothelin 1; **HRT:** hormone replacement therapy; **I/R:** ischemia-reperfusion; **LDL:** low density lipoprotein; **LVH:** left ventricular hypertrophy; **MCIP:** modulatory calcineurin-interacting protein; **MISS:** membrane-initiated steroid signaling; **PI3K:** phosphotidylinositol-3 kinase; **PKC:** protein kinase C; **siRNA:** small interfering ribonucleic acid | 

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## Introduction

Estrogen exerts complex biological effects through its cognate receptors. There are two isoforms of estrogen receptors (ERs): ERα and ERβ, which are encoded by distinct genes with different levels of expression in various tissue types [Nilsson and Gustafsson, 2002]. Both are found in endothelium of human blood vessels and in the myocardium [Grohe et al., 1997; Karas et al., 1994; Kim-Schulze et al., 1996]. As members of the nuclear receptor superfamily, ERs positively regulate gene expression by binding to target gene estrogen response elements, modifying the binding of other transcription factors, and recruiting co-activators to the transcriptosome. ERs also participate in rapid, plasma membrane-initiated steroid signaling (MISS), which in part impacts transcription. Whether through alteration of gene expression or through protein modification by rapid signaling, estrogen-activated ERs significantly modulate cardiovascular physiology. This article focuses on multiple actions of estrogen and ligand-dependent ERs relevant to the cardiovascular system.

## Role of estrogen in vasculature

Estrogen has often been shown to prevent vascular dysfunction and injury. 17-β-estradiol (E2) accelerates endothelial recovery and increases nitric oxide (NO) production after de-endothelializing balloon injury [Krasinski et al., 1997]. This is ER-dependent, as shown in studies utilizing a specific ER inhibitor, ICI182780, and is absent in ERα knockout (KO) mice [Bakir et al., 2000; Karas et al., 2001]. Increased NO production induced by E2 results in vasodilation of the coronary microvasculature [Thompson et al., 2000], carotid and femoral arteries [Guo et al., 2005]. Recent data derived from comparison of wild type, ovariectomized, and eNOS-null mice show that expression of endothelial nitric oxide synthase (eNOS) is significantly reduced in ovariectomized mice. Furthermore, eNOS is induced by E2 [Lantin-Hermoso et al., 1997], and this interaction relieves stress and strain on the arterial wall [Guo et al., 2006]. Rapid activation of eNOS by E2 occurs in minutes, and suggests non-transcriptional actions or MISS [Caulin-Glaser et al., 1997; Chen et al., 1999]. E2-mediated eNOS activation results from rapid activation of phosphotidylinositol-3 kinase (PI3K)/Akt, demonstrated in cultured human endothelial cells (EC) [Haynes et al., 2000] as well as in intact elastic and muscular arteries *in vivo* [Guo et al., 2005; Haynes et al., 2000]. E2-mediated vasodilation is also absent in eNOS KO mice, confirming the interaction between E2/ER and eNOS in influencing the vascular tone [Guo et al., 2005].

Direct binding of ERα to the p85 subunit of PI3K and increased PI3K activity (resulting in activation of Akt and eNOS) has been reported to occur in an E2-dependent fashion, blocked by estrogen receptor antagonists ICI182780 and tamoxifen [Simoncini et al., 2000]. In addition, E2-dependent rapid activation of eNOS occurs via an interaction between ERα and the $G_{\alpha i}$-protein [Wyckoff et al., 2001].

Does membrane-localized ERα have the ability to function as a G-protein-coupled receptor (GPCR)? The structure of ERα is much different from that of classical GPCRs, and endogenous ERα is unlikely to span the plasma membrane of cells. However, collective evidence (e.g. membrane localization, activation of signaling cascades, association with G-proteins) implicates that ERα can participate in MISS via G-protein activation to downstream signaling [Kelly and Levin, 2001]. In some situations, this could involve membrane ER or E2-activation of an orphan GPCR, like GPR30 [Revankar et al., 2005], but this is controversial.

Although ERα alone was sufficient to bind and activate PI3K in the study by Simoncini et al., it does not preclude a potential recruitment of ERβ to form heterodimers that enact signaling. Chambliss and colleagues demonstrated rapid signaling actions of ERβ at membrane-associated caveolae [Chambliss et al., 2002]. Furthermore, work from our own laboratory indicates that plasma membrane ERs function as dimers to bind and activate G-protein subunits. While most endogenous plasma membrane ERs exist as homodimers in the presence of E2, a small portion of the receptor pool forms ERα/ERβ heterodimers [Razandi et al., 2004]. We also reported that *in vivo* kinase activation and arterial vasodilator responses to E2 are absent in either ERα or ERβ KO mice, indicating that both ER isoforms cooperate to impact vascular functions [Guo et al., 2005]. We believe that the *in vitro* data (demonstrating the membrane localization of both ER isoforms and the existence of heterodimers) complement our *in vivo* model utilizing the isoform-specific null mice. Together, they raise an interesting possibility that ERα/ERβ heterodimers at the membrane may be involved in modulating eNOS activation and vasodilation. It also should be noted that no study, to our knowledge, has proved *in vivo* that homodimers are sufficient for PI3K activation. However, details about how the existing heterodimers in MISS differ functionally from a majority pool of homodimers remain largely unknown. In order to isolate the contribution of heterodimers from homodimer isoforms, we may consider a systematic approach of mutagenesis, first to define the necessary residues important for forming heterodimers, and see if they differ from those needed for homodimerization. Once more information is known for heterodimer interaction, further functional studies comparing homodimers and heterodimers may be possible.

Vasoprotection by E2 is also modulated by nuclear ERs interacting with co-activators. Transgenic mice lacking the steroid receptor coactivator-3 (SRC3), a protein highly expressed in smooth muscle cells and EC, exhibit pronounced neointimal proliferation. Furthermore, female mice are less responsive to the inhibitory effect of E2 on this proliferation [Yuan et al., 2002]. A newly identified co-repressor of ERα, MRF1 (Modulator Recognition Factor 1), richly expressed in cardiovascular tissues, inhibits transcriptional activation by ERα, binds ERβ, and may be a good candidate to provide modulation of ER actions on the vasculature [Georgescu et al., 2005].

Another major effect of E2 in the vasculature is the inhibition of atherosclerosis [Mendelsohn and Karas, 1999]. Proposed mechanisms include anti-inflammatory actions, reduction of LDL oxidation, and promotion of re-endothelialization [White, 2002]. Double KO mice deficient for ERα and apolipoprotein E (Apo-E) indicate that ERα is a major mediator of the atherosclerosis reduction [Hodgin et al., 2001]. Apo-E KO mice ($ApoE^{-/-}$) typically develop lipid-induced atherosclerosis and provide a good experimental model of this disorder. $ApoE^{-/-}$ mice, when treated with E2, exhibit 80% less lesions, while such benefit is abrogated in mice lacking both ERα and ApoE. By contrast, E2 treatment reduces atherosclerotic progression equally in $ApoE^{-/-}$ mice and in ERβ-deficient $ApoE^{-/-}$ mice. This finding indicates that the ERα isoform is the main mediator of atherosclerosis mitigation, and the underlying mechanism includes upregulation of the athero-protective prostacyclin, PGI2, by ERα [Egan et al., 2004].

While ERβ does not appear to alter atherosclerotic progression significantly, it affects multiple aspects of vascular integrity in addition to modulating eNOS-dependent vasodilation [Guo et al., 2005; Watanabe et al., 2003; Zhu et al., 2002]. For example, vascular smooth muscle cell proliferation is strongly inhibited by ERβ [Watanabe et al., 2003]. Furthermore, deleting this ER subtype in mice results in ion channel abnormalities of vascular smooth muscle cells, attenuation of endothelium-independent iNOS production and subsequent vasoconstriction, and the development of both systolic and diastolic hypertension [Zhu et al., 2002]. Related to the issue is a possibility that E2/ERs can mitigate abnormal ventricular hypertrophy of the heart, commonly resulting from long-standing hypertension.

## Effects of estrogen on the myocardium

Left ventricular hypertrophy (LVH) of the heart is an independent risk factor for adverse cardiovascular outcomes such as stroke and heart failure [Gosse, 2005]. It has been noted that pre-menopausal women have a lower prevalence of LVH than age-matched men [Agabiti-Rosei and Muiesan, 2002], and that hormone replacement therapy (HRT) with E2 reverses LVH in postmenopausal women [Miya et al., 2002; Modena et al., 1999]. Animal studies also support the anti-hypertrophic actions of E2 [van Eickels et al., 2001]. Complementary mechanistic studies indicate that attenuation of LVH is not just an indirect sequela of E2-related afterload reduction. E2 efficiently reduces angiotensin II (AngII)- or endothelin 1 (ET1)-induced hypertrophy of individual cardiomyocytes, resulting in a smaller cell surface area and decreased cytoskeletal protein expression [Pedram et al., 2005].

Detailed *in vitro* data suggest that underlying mechanisms are multifold and entail the following [Pedram et al., 2005]. E2 blocks AngII- or ET1- initiated hypertrophic signaling, in part through activation of PI3K. A notable downstream event is upregulation of the gene encoding modulatory calcineurin-interacting protein (MCIP), a calcineurin antagonist. E2 stimulates both the transcriptional

transactivation and mRNA stability of the MCIP gene. Inhibition of calcineurin phosphatase activity by MCIP subsequently prevents nuclear translocation of a family of transcription factors, called nuclear factor of activated T cells (NF-AT). This prevents NF-AT transcriptional activity and subsequent stimulation of the cardiac hypertrophic gene response. In addition, E2 antagonizes AngII-induced extracellular signal-regulated kinase (ERK) and protein kinase C (PKC), two modulators central to hypertrophic signaling [Braz et al., 2002]. Inhibition of these two kinases by E2/ERs involves the upregulation of atrial natriuretic peptide (ANP) production and appears independent of interaction with MCIP. However, crosstalk between the calcineurin-NFAT pathway and PKC-ERK signaling exists in cardiomyocytes [Pedram et al., 2005; Sanna et al., 2005], and may provide the basis for E2/ERs to achieve the overall antihypertrophic effect at multiple points of the interrelated pathways. Therefore, E2/ERs mitigate cardiac hypertrophy in this model via an integrative fashion involving actions at MISS and transactivation of the involved genes. We believe that E2-activated PI3K signaling to MCIP transactivation provides an example of MISS signaling to transcription in cardiomyocytes [Pedram et al., 2005].

As observed in both the vascular function and effects on LVH, association of ERs with PI3K plays an important protective role in ischemia-reperfusion (I/R) injury to the heart. I/R injury is strongly linked to cardiomyocyte loss via apoptosis, probably due to a combination of a significant ischemic burden and oxidative stress from reperfusion. In an animal model of myocardial infarction by permanent coronary occlusion, E2 protection of the heart includes reducing infarct size and decreased myocyte apoptosis due to activation of the PI3K/Akt pathway [Patten et al., 2004]. Similar E2-mediated signaling may protect cardiomyocytes from I/R-related injury, including apoptosis. Indeed, a simulated I/R model *in vitro* from our laboratory shows that E2-mediated reduction of cardiomyocyte apoptosis involves activation of PI3K/Akt [Kim et al., 2006].

In general, there are two types of apoptosis: a “death-receptor”-driven extrinsic pathway and a mitochondria-centered intrinsic pathway. It is not completely known where in the apoptotic signaling E2/ER intervenes, and data regarding E2 actions on apoptosis in the heart are still limited. Nevertheless, several known features of estrogen are relevant to the cytoprotective mechanism of E2 in the cardiomyocyte. For example, E2 serves as an antioxidant in differentiated cells such as neurons. Oxidative stress and the subsequent generation of reactive oxygen species (ROS) following coronary reperfusion are thought to be a potent trigger for cardiomyocyte apoptosis [Zhao, 2004]. The ability of E2/ERs to counteract such redox intermediates is likely to be a key component of the overall protection. In addition, E2 modulates yet another important kinase participating in the cell fate upon stress: p38 MAPK. Of the known p38 isoforms (α, β, δ, and γ), p38α is a predominant form expressed in the heart, upregulated in ischemia, and considered to induce apoptosis. On the other hand, p38β (also found in cardiomyocytes) is often anti-apoptotic in function. Importantly, E2 modulates the two isoforms differentially, inhibiting p38α and stimulating p38β. *In vitro* data from our laboratory using cultured cardiomyocytes and simulated I/R suggest that E2 activation of PI3K is linked to ROS suppression and p38β upregulation. This signaling leads to p38α downregulation and cell survival [Kim et al., 2006]. By modulating these key signaling molecules in the apoptotic pathway, E2/ER efficiently reduces cell death.

Which ER is responsible for cardioprotection from I/R? The two isoforms have differential intracellular localization upon ligand activation (ERα is mainly translocated to the nucleus with some receptors at the plasma membrane, in contrast to ERβ seen mainly in mitochondrial/perinuclear areas as well as the membrane [Pedram et al., 2005; Yang et al., 2004]). *In vivo* models of myocardial infarction and heart failure utilizing either ERα or ERβ knockout mice support the conclusion that both ERα and ERβ are essential in E2-related protection of cardiomyocytes from an ischemic insult [Booth et al., 2005; Gabel et al., 2005; Pelzer et al., 2005; Zhai et al., 2000]. This may result in part from signaling by ERα/β heterodimers [Li et al., 2004], either in the nucleus or at the plasma membrane [Guo et al., 2005]. How ERs acting as heterodimers might intercede in apoptotic signaling is not clear at this point.

## Conclusion

Estrogen acting through ERs confers critical protection to the cardiovascular system by its pleiotropic effects in signaling. E2-mediated outcomes include several important physiological adaptive responses: anti-atherogenic actions, vasodilation and preservation of vascular integrity, alleviation of left ventricular hypertrophy, and cardiomyocyte survival by preventing apoptosis.

Despite much human and animal experimental data, doubt was cast regarding the beneficial effects of estrogen when HRT was tested as a primary intervention for atherosclerotic heart disease in the Women’s Health Initiative trial (WHI) [Rossouw et al., 2002]. However, the conclusions from the trial have been challenged on several bases [Mendelsohn and Karas, 2005; Turgeon et al., 2004]. For example, the trial methodology overlooks the critical relationship between the timing of HRT initiation and the natural history of atherosclerosis in women. The study population was post-menopausal at a mean age of 63, and had more than a decade of estrogen deficiency at enrollment. Furthermore, the majority of subjects were obese, predisposing them to atherosclerotic heart disease during the period of estrogen deficiency. Thus, many of the women at the initial screening already had features associated with atherosclerosis in this trial designed for *primary* prevention. In addition, the results derived from the use of the specific hormone formulation in the trial may not be applicable to other HRT available. Currently, optimized clinical trials are under way to clearly define the long-term effects of early intervention HRT on arteriosclerosis. In

the meantime, the cumulative body of evidence demonstrating potent beneficial effects of E2 on the heart and vasculature warrant continued research efforts to investigate the mechanisms and develop related therapeutic intervention.

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