Brief Reviews

Nonnuclear Actions of Estrogen

Karen J. Ho, James K. Liao

Abstract—Estrogen has long been observed to endow cardiovascular protective effects, as evidenced by sex-specific differences in the incidence of hypertensive and coronary artery disease, the development of atherosclerosis, and myocardial remodeling after infarction. To exert its tissue-specific effects, the classic estrogen receptor (ER) functions as a ligand-dependent transcription factor. However, there is growing evidence that in response to 17β-estradiol and heterologous signals, the ER can also mediate signaling cascades at the membrane and in the cytoplasm via various second messengers, such as receptor-mediated protein kinases. This review summarizes the current understanding of nonnuclear ER signaling and discusses the relevance to eliciting the beneficial cardiovascular effects of estrogen. These include vasodilation, inhibition of response to vessel injury, limiting myocardial injury after infarction, and attenuating cardiac hypertrophy. Defining the full repertoire of ER function promises to expose novel, highly specific targets for pharmacological interventions and may ultimately lead to the primary and secondary prevention of cardiovascular diseases. (Arterioscler Thromb Vasc Biol. 2002;22:1952-1961.)

Key Words: estrogen ■ estrogen receptors ■ transcription ■ vasculature ■ signaling

Sex-based differences in the incidence of hypertensive heart disease and coronary artery disease, the development of atherosclerosis, and cardiac remodeling after myocardial infarction have long been observed.1–3 In addition to improving risk factors, such as the lipid profile, estrogen also has direct effects on the myocardium, endothelium, and vascular smooth muscle. Although the estrogen receptor (ER) is classically a ligand-dependent transcription factor, it is becoming apparent that the receptor also modulates the activity of intracellular second messengers and membrane-associated receptors and signaling complexes, some of which can also enhance the classic activity of the ER. In the heart and vasculature, these nonnuclear signaling pathways mediate rapid vasodilation,4 inhibition of the response to vessel injury,5–10 reduction in myocardial injury after infarction,11,12 and attenuation of cardiac hypertrophy.13,14

ER Structure and Function

The binding of 17β-estradiol (E2) to the ER initiates a myriad of possible signal transduction pathways that, depending on the cellular context, elaborate responses as varied as survival, adhesion, and proliferation and culminate in physiological processes as divergent as cardiovascular protection, bone preservation, organogenesis, and cancer. The 2 subtypes of ER, ERα and ERβ, are synthesized from separate genes and are structurally and functionally distinct. Both subtypes are classic steroid hormone receptors and are members of the nuclear receptor superfamily.15,16 The 5 steroid hormone receptors, constituting class I of the superfamily, share the same modular organization of a ligand-binding domain, DNA-binding domain, and 2 transcriptional activation function domains (Figure 1A). A central feature of classic ER action is ligand-dependent regulation of gene expression in target tissues.1,2,17 Binding of estrogen to ER releases the receptor from an inhibitory complex with heat shock proteins, leading to homodimerization and translocation of the receptor complex into the nucleus. The ER then binds to a 15-bp palindromic sequence called the estrogen response element (ERE), located in the promoter region of target genes. Maximum transcriptional activity requires the concerted actions of the ligand-independent activation function (AF)-1 domain (an area of site-specific phosphorylation) in the amino terminus and the ligand-dependent AF-2 in the carboxy terminus. Together, they recruit a coregulator complex to the promoter; the tissue, cell, and promoter–specific complex components expose the transcriptional template, resulting in transactivation or transrepression.18,19

The cardiovascular importance of estrogen has been probed with receptor gene deletion or mutation studies20 (Figure 1B). A young man with a homozygous disruption in the ERα gene resulting in the expression of a truncated receptor lacking DNA and hormone-binding domains developed premature coronary artery disease and impaired brachial endothelium-dependent vasodilation.21,22 However, this is only a single case study and should be viewed with caution because other genes may also be affected. Early studies in ovariecotomized mice demonstrated that E2 inhibits intimal and medial vascular smooth muscle proliferation,9 suggesting a direct protective effect of estrogen on endothelial and vascular smooth muscle cells (VSMCs). In subsequent ca-
rotid injury studies, E2 inhibited medial thickening and VSMC proliferation in wild-type and ERα knockout (ERαKO) mice, implying that the protective effect of E2 could be mediated in an ERα-independent manner. Furthermore, in ERα and ERβ double-knockout mice, E2 inhibited only VSMC proliferation, suggesting instead that a retained splice variant of ERα that lacked only the amino-terminal activation function domain could mediate partial protection. This quandary was resolved with the production of complete ERα null mice, which exhibit increased medial area, VSMC proliferation, and deposition of proteoglycans in response to vascular injury. Similarly, hearts from ERαKO mice subjected to global ischemia and reperfusion exhibit greater global ischemia and a higher incidence of arrhythmias. Hearts from ERαKO mice also have higher calcium accumulation, implicating that E2 inhibits calcium influx and attenuates the harmful effects of calcium overload during myocardial ischemia/reperfusion. The mechanism of these effects may involve NO, which ameliorates coronary dysfunction and reduces tissue edema by decreasing microvascular permeability, inasmuch as hearts from ERαKO mice demonstrate decreased NO release. ERα also mediates the neuroprotective effects of E2 after cerebral ischemia, as demonstrated by greater stroke sizes in ovariectomized ERαKO mice subjected to permanent cerebral ischemia.

In addition, there is growing evidence that ERβ may also have an important function in the vasculature. ERβ expression is induced in VSMCs after vascular injury, and ERβ knockout mice exhibit hypertension and ion channel dysfunction in VSMCs.

This review, however, will focus on ERα, given the greater body of work available. Discerning the components of the rapidly expanding ER signaling network and understanding its potential role in disease states may provide new opportunities for highly context-specific therapeutic strategies.

Nonnuclear Actions of Estrogen

Our appreciation of the potency and versatility of ERα signaling is growing in light of accumulating evidence that ERα can also elicit rapid cellular effects that peak minutes after stimulation in multiple cell types (Figures 2 and 3). Given that the rapidity of activation makes modulation of gene transcription less likely and that the effects are not blocked by inhibitors of protein or RNA synthesis, these extranuclear mechanisms are commonly referred to as “nonnuclear” or “nongenomic” effects of estrogen. These signaling cascades recruit second messengers including calcium and NO, receptor tyrosine kinases including epidermal growth factor (EGF) receptor and insulin-like growth factor (IGF)-1 receptor, G-protein–coupled receptors (GPCRs), and protein kinases including phosphoinositide-3 kinase (PI3K), serine-threonine kinase Akt, mitogen-activated protein kinase (MAPK) family members, nonreceptor tyrosine kinase Src, and protein kinases A and C (see reviews17,33–42; Figure 2).

Because many of these estrogen-stimulated pathways are typically initiated at the plasma membrane, many investigators have sought to determine the existence of a membrane-associated ER. Indeed, membrane binding sites for E2 were first implicated in 1977, but the precise nature of the receptor remains elusive. Examination of the structure of ERα further increases the controversy surrounding the existence of a membrane receptor. For example, ERα possesses neither intrinsic kinase nor phosphatase activity, does not have hydrophobic stretches that could represent transmembrane domains, and lacks myristoylation and palmitoyl-
ation sequences that could anchor it to the membrane. To date, indirect evidence of a membrane ERα comes from immunohistochemistry and from studies with membrane-impermeable ligands or overexpressed nuclear receptors.

Studies with E2, which has been conjugated to BSA or to fluorescent macrocomplexes, suggest that a small population of cellular ERα may be localized to the cellular membrane, inasmuch as both membrane-impermeable forms...
elicit the same rapid effects as unconjugated E2. Although contamination with unlabeled ligand is a possible confounding factor, E2-BSA competes with unlabeled E2, tamoxifen, and ERα antibody for binding to the cell membrane and enters the cytoplasm only when the cells are permeabilized.55 E2-BSA also does not activate ERE-dependent transcription, again suggesting that the compound remains extracellular.52,53 Finally, the nonnuclear cascades observed with E2-BSA stimulation are not inhibited with the intracellular pure ER antagonist ICI 182,780.52 Of particular relevance to the vascular system is the observation of a membrane receptor in endothelial cells (ECs) that binds either E2 or E2-BSA rapidly and selectively activates antiapoptotic p38β MAPK and inhibits proapoptotic p38α, leading to upregulation of MAPK-activated protein kinase-2 kinase and phosphorylation of heat shock protein hsp27.56 Downstream effects of these effects include preservation of stress fiber formation and membrane integrity, prevention of hypoxia-induced apoptosis, and induction of both EC migration and the formation of primitive capillary tubes. Thus, estrogen may exploit these effects in neuronal and vascular systems.58 Furthermore, vascular and cardiac protection model systems, the liganded ERα associates with the p85α regulatory subunit of PI3K in a ligand-dependent manner.60 Alternatively, rapid E2-mediated vasorelaxation may occur in an endothelium-independent manner by changes in calcium flux in VSMCs.65

The presence of chaperone hsp90 upregulates eNOS activity in ECs,66 and hsp90 inhibitors disrupt the E2-induced hsp90-eNOS association.57 Indeed, the hsp90 inhibitor geldanamycin, the PI3K inhibitor LY294002, and the eNOS inhibitor No-nitro-l-arginine methyl ester inhibit E2-dependent vasodilation of rat aortic rings.68 Because hsp90 interacts with eNOS and Akt and modulates eNOS activity, hsp90 likely functions as a scaffold to regulate Akt-dependent phosphorylation of eNOS.69

Nonnuclear recruitment of MAPK signaling cascades by ERα has also been demonstrated. Activation by estrogen of extracellular signal–regulated kinase (ERK)-1/2 has been shown in cardiomyocytes,70 colon cancer,71 breast cancer,72 and bone,73,74 leading to responses that include cell growth, cell cycle progression, and survival. The antiproliferative effects of estrogen in VSMCs75 and lung myofibroblasts,76 on the other hand, are mediated by inhibition of ERK-1/2. Interestingly, by activating MAPKs in a nonnuclear manner, ERα may be amplifying its classic function as a transcription factor. For instance, E2 rapidly activates ERK-1/2 in lactotroph cells; this activation upregulates prolactin gene transcription, a mechanism of activity that occurs in parallel with direct ERα activation of the prolactin gene by classic ERE-dependent transcriptional activation.77 Nonnuclear ERα activity can also give rise to ERE-independent transcriptional activation. In human neuroblastoma cells, E2-BSA induces the transcription of a reporter gene construct driven by the promoter of the immediate-early gene c-fos.52 In addition, E2 rapidly increases the expression of early growth response gene-1 in cardiac myocytes by inducing the recruitment of serum response factor to serum response elements in the early growth response gene-1 promoter via ERK-1/2.78

Rapid activation of more proximal kinases may be the mechanism for the activation of ERK by estrogen. In overexpression systems, the liganded ERα induces rapid phosphorylation of the IGF-1 receptor and activation of ERK-1/2. Indeed, the 2 receptors communoprecipitate in a ligand-dependent manner, suggesting a direct physical interaction between ERα and the IGF-1 receptor.79 In breast cancer cell lines, ERαs induce rapid phosphorylation of the adaptor proteins, Src and Shc, in a ligand-dependent manner, resulting in an Shc–growth factor receptor binding protein (Grb)-2–son of sevenless (SoS) complex formation.80 This leads to the subsequent activation of Ras, Raf, and MAPK. Similarly, in breast and prostate cancer cells, E2 treatment activates the Src-Ras-ERK pathway, leading to cell cycle progression.81,82 In these studies, direct interaction between phospho-Tyr537 of ERα and the Src homology domain 2 activates Src activity. In cortical neurons subjected to glutamate toxicity, estrogen also rapidly activates Src family tyrosine kinases and tyrosine phosphorylation of Ras, leading to neuroprotection.83 Furthermore, rapid phosphorylation of Src has also been observed in osteoclasts, although the ramifications for bone resorption remain to be defined.84 Interestingly, in osteoblasts, osteocyttes, and embryonic fibroblasts, activation of an Src-Shc-ERK signaling pathway prevents apoptosis.85 Finally, in breast cancer cells, Src modulates PI3K-Akt signaling by a reversible cross-talk mechanism in which ligand binding induces the formation of a ternary complex between ERα, PI3K, and Src.86 Cross talk between PI3K and Src has also been observed in osteoclasts87 and bone marrow cells.88 Whether a similar complex plays a role in eNOS activation in ECs remains to be determined.
Tissue-Specific Effects of Selected SERMs

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<td>Tamoxifen</td>
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<td>Total cholesterol lowering, no effect on HDL 132</td>
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<td>GW5638</td>
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<td>EM-800</td>
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<td>–136</td>
<td>Total cholesterol and triglycerides lowering 136</td>
<td>NO-mediated vasodilation 136</td>
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<tr>
<td>Raloxifene</td>
<td>–151</td>
<td>–137</td>
<td>Total cholesterol and triglycerides lowering, no effect on HDL 138</td>
<td>NO-mediated vasodilation 140-142 ROS reduction 141</td>
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<td>LY117018</td>
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<td>–152</td>
<td>Total cholesterol lowering 155</td>
<td>NO-mediated vasodilation 139</td>
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– signifies antagonist activity; +, agonist activity.

In addition to recruiting ERK-1/2, ERα also modulates other MAPK family members. ERα in the heart selectively activates MAPK cascades to modulate the development of cardiac hypertrophy.8,11,14,89 For example, mice were protected from pressure-overload hypertrophy by ERα-mediated selective inhibition of p38 MAPK.89 Apparently, ERK and c-Jun N-terminal kinase (JNK) are not involved,90 consistent with recruitment of p38 in other models of cardiac hypertrophy.91-92 In breast cancer cells stably transfected with ERα and resistant to the anti-estrogen tamoxifen, loss of estrogen-mediated activation of p38 MAPK is correlated with survival.93 In ERα-positive breast cancer cell lines, however, activation of JNK promotes survival from taxol-induced or ultraviolet radiation-induced apoptosis.94 Finally, induction of eNOS and inducible NOS in cardiac myocytes is blocked by the MAPK inhibitor PD98059,70 which may have clinical relevance because NO inhibits caspase activation and prevents the development of congestive heart failure.94

E2-Independent Nonnuclear Activity Potentiates AF-1 Function

The nonnuclear ERα activity has been shown to enhance the nuclear activity of the receptor in the context of E2-independent activation of the receptor. Indeed, ERα integrates a variety of heterologous signals, including dopamine,95,96 serum,97 cAMP,98 cavelolin,99,100 and cyclins A and D.101-104 Activation by EGF and IGF-1 provides the best example of modulation of ERα nuclear activity by nonnuclear E2-independent stimulation. Through this cross-talk mechanism, mitogenic extracellular signals are translated into cell cycle progression or, in cancer cells, into proliferation in the absence of hormone.105 EGF-stimulated and IGF-1-mediated stimulation of MAPKs results in the direct phosphorylation of ERα on Ser118.73,106,107 Phosphorylation of ERα enhances the binding of p68 RNA helicase108 and accounts for enhanced AF-1 transcriptional activity in uterine and ovarian adenocarcinoma cells.109-111

In addition to direct phosphorylation of the receptor, EGF can also modulate the coactivator phosphorylation state. Steroid receptor coactivator-1, a member of the p160 family of adaptor molecules that recruit other proteins to the coactivator complex, contains consensus sequences for ERK-1/2, and EGF stimulation results in ERK-1/2-mediated phosphorylation of steroid receptor coactivator-1, which potentiates ERα transcriptional activity.112 Alternatively, EGF or IGF-1 stimulation can activate the PI3K-Akt pathway, which in turn, activates E2-responsive target genes. In breast cancer cell lines, EGF or IGF-1 treatment cause rapid phosphorylation and activation of Akt, leading to increased levels of progesterone receptor mRNA and protein.113 All of these effects were blocked by the PI3K inhibitor, wortmannin, and ICI 182,780 and were mimicked in the presence of a constitutively active Akt mutant. Akt may also activate ERα by phosphorylation of Ser167 within the AF-1 domain.113 Interestingly, ERα binds constitutively to the p85α subunit of PI3K and activates PI3K/Akt in an E2-independent manner, implicating a feed-forward mechanism of ERα activation.114 Finally, nonreceptor tyrosine kinase Src, in addition to modulating E2-dependent nonnuclear activities of ERα in the setting of mitogen and PI3K stimulation, may influence the transcriptional activity of ERα in an E2-independent manner. In cells overexpressing ERα and v-Src, Src stimulates ERα transcriptional activity by enhancing AF-1 function via 2 parallel cascades. In the first instance, an Src–Raf-1–mitogen-activated ERK kinase–ERK pathway leads to phosphorylation of Ser118 in the AF-1 domain.113 In the same cells, a second pathway mediated by Src, mitogen-activated ERK kinase kinase, JNK kinase, and JNK may indirectly activate transcription by modulating AF-1–associated coactivators.115 Although these studies have implications for the role of Src in tumor progression, it is also interesting to speculate whether there could be a feedback mechanism by which nonnuclear activation of Src by ERα enhances ERα transcriptional activity.

Membrane Origin of Nonnuclear ER Activity

The trafficking of ERα to different cellular compartments may be regulated by the nature of stimulation. In VSMCs transfected with ERα, MAPK activation mediates nuclear translocation of ERα from the membrane by E2-dependent and -independent mechanisms.116 Another proposed mechanism for membrane-initiated signaling by ERα involves receptor association with membrane caveolae, which are cholesterol-rich membrane domains containing signaling molecules such as G proteins, GPCRs, PKC, receptor tyrosine kinases (RTKs), and non-RTKs. In fractionated EC plasma membranes, ERα protein has been localized to caveolae, and E2 stimulates eNOS in isolated caveolae in an ERα- and calcium-dependent manner.117-119 The close association of ERα with caveolae and the regulation of eNOS phosphorylation and activity with hsp90 suggest an additional mechanism of action, inasmuch as cavelolin-1 (cav-1), the coat protein for caveolae, and hsp90 independently coimmunoprecipitate with eNOS in EC lysates.120 Indeed, hsp90–eNOS–
cav-1 may exist in a heterotrimeric complex in ECs such that the cav-1 scaffolding peptide is inhibitory and, on increase in cytoplasmic calcium, calcium-activated calmodulin may aid in the further recruitment of hsp90 to the complex by facilitating the release of the caveolin from eNOS. In vivo confirmation has been obtained by systemic administration of a chimeric peptide containing the cav-1 scaffolding peptide to mice. The protein was taken up by ECs and suppressed NO production and acute inflammation.

Nonnuclear ERα signaling also involves membrane heterotrimeric G proteins. For example, in Chinese hamster ovary cells transfected with ERα cDNA, membrane and nuclear-localized receptors are detected. ERα in the membrane fractions activated Goα and Gqα and rapidly stimulated inositol phosphate production and adenylyl cyclase activity, respectively. Alternatively, G-protein activation has also been shown in ECs, where E2 activation of eNOS can be inhibited with ICI 182,780, RGS-4 (a regulator of G-protein signaling specific for Goα and Gqα), and pertussis toxin (specific for Goα). In communoprecipitation studies, ERα interacted with Goα but not Goα or Gqα in a ligand-dependent manner, whereas pertussis toxin completely blocked this interaction.

Are There Other ER Isoforms?
Nonnuclear signaling, alternatively requires a GPCR that is distinct from ERα. Indeed, in macrophage cell lines, E2 and E2-BSA induced a rise in intracellular calcium that was inhibitable with pertussis toxin, and sequestration of a E2-GPCR occurred independently of chlathrin-caveolin pathways. An E2-GPCR has also been postulated to exist in the hippocampus, where E2 stimulation potentiates kainate-induced currents through the modulation of protein kinase A activity.

Recent evidence suggest that the nonnuclear effects of estrogen are, in fact, mediated by a receptor distinct from ERα or ERβ. For example, in the cerebral cortex, estrogen rapidly stimulates tyrosine phosphorylation of c-Src, which then induces phosphorylation of Shc and Shc-growth factor receptor binding protein-2 complex formation, which is upstream from ERK and B-Raf activation. Coincidentally, hsp90 coimmunoprecipitates with ERK-1/2 and may either preserve its conformation for subsequent phosphorylation with mitogen activated ERK kinase-2 or protect the phosphorylated kinase from phosphatases. Surprisingly, however, the pathway is intact and not inhibitable by ICI 182,780 in ERαKO cortical explants, and ERα- and ERβ-selective ligands fail to reproduce the effects in KO cells. Taken together, these data suggest that a novel receptor, responsive to E2 but insensitive to ICI, mediates nonnuclear neurite differentiation.

There is considerable controversy regarding the nature of the ERα that mediates the nonnuclear effects of estrogen. Further studies using truncation mutants of ERα or cells cultured from complete null ERαKO mice may help identify the receptor or the domains of the ERα that are responsible for these effects.

Implications for SERM Development
Nonetheless, the central role of the ER signaling network in cancer, cardiovascular disease, osteoporosis, and neurological disease and an increasingly detailed understanding to the underlying cell biology have made ER an attractive target for pharmacological intervention. Selective estrogen receptor modulators (SERMs) are ER ligands that can have varying agonist or antagonist activities given the cell, promoter, and coregulator context.

Tamoxifen, the prototypical SERM, is a triphenylethylene that, because of its agonist activity in the liver, reduces serum total cholesterol and LDL levels. Unfortunately, its strong agonist activity in the endometrium leads to endometrial hyperplasia and low-grade cancers. GW5638, a derivative of tamoxifen, shows some promise in early animal studies, inasmuch as it possesses estrogenic activity in preserving bone and lowering serum cholesterol while lacking agonist activity in the uterus.

EM-800, a nonsteroidal compound, is the active form of EM-652 and demonstrates higher affinity for ERα compared with E2, tamoxifen, or any other SERM. In addition to possessing potent antitumor activity in the uterus and breast, EM-800 prevents bone loss and lowers serum cholesterol and triglyceride levels. Furthermore, in vitro studies in ECs suggest that EM-800, like E2, enhances NO release by sequential activation of MAPKs and PI3K-Akt, implicating an additional vascular protective effect.

Raloxifene, which is also a nonsteroidal compound, is similar to tamoxifen in activity although it is less agonistic in the endometrium. Raloxifene is administered primarily for bone preservation. Regarding its effects on the vasculature, raloxifene reduces serum triglycerides and serum fibrinogen levels. Raloxifene and its analogue, LY117018, stimulate eNOS activity in ECs via PI3K and ERK-dependent pathways, respectively. They have also been shown to inhibit the release of reactive oxygen species from smooth muscle cells. Accordingly, raloxifene treatment induces coronary artery relaxation in an ERα- and NO-dependent manner. It also improves endothelium-dependent vasorelaxation in hypertensive rats by enhancing the expression and activity of NO synthase.

The differential actions of estrogen and SERMs suggest complex regulatory mechanisms for suppression and activation in a context-specific manner. These mechanisms depend on the ligand, the promoter of the target gene, and the combination and exchange of coregulators. Of clinical interest, breast cancer and pituitary lactotroph tumors demonstrate enhanced apoptosis and tumor shrinkage when they are transfected with adenovirus constructs containing dominant-negative ERα mutants. Given evidence that dominant-negative ERα and anti-estrogens recruit transcriptionally repressive proteins to their DNA-binding complex that enhance their antagonistic activity, the precise regulatory proteins that govern ERα activity in other disease states represent promising therapeutic strategies.

Summary
We are at the threshold of understanding the full repertoire of ER action. Although the steroid receptor signaling field has
made significant strides in defining its intertwining modes of action in numerous tissue types, from the nucleus to the cytoplasm and perhaps to the plasma membrane, a full understanding of how ER functions in physiological and pathophysiological states remains to be determined. Recent data from the Heart and Estrogen/Progesterin Replacement Study (HERS) II trial,148 suggesting no cardiovascular benefit from extended hormone replacement therapy, underline the importance of isolating the nonnuclear mechanisms of estrogen action and delving deeper into the modulation of ER transcriptional activity by coregulators. Only after we develop a detailed understanding of these highly cell- and promoter-specific mechanisms can they be exploited for formulating clinically meaningful treatment strategies for the primary and secondary prevention of cardiovascular diseases in men and women.

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