Selective Agonists of Estrogen Receptor Isoforms
New Perspectives for Cardiovascular Disease

Chiara Bolego, Elisabetta Vegeto, Christian Pinna, Adriana Maggi, Andrea Cignarella

Abstract—The cloning of estrogen receptors (ERs) and generation of ER-deficient mice have increased our understanding of the molecular mechanisms underlying the cardiovascular effects of estrogen. It is conceivable that clinical trials of estrogens so far failed to improve cardiovascular health because of the poor ER isofrom selectivity and tissue specificity of endogenous hormones as well as incorrect treatment timing and regimens. Tissue-selective ER modulators (SERMs) may be safer agents than endogenous estrogens for cardiovascular disease. Yet, designing isoform-selective ER ligands (I-SERMs) with agonist or antagonist activity is required to pursue improved pharmacological control of ERs, especially taking into account emerging evidence for the beneficial role of vascular ERα activation. Ideally, the quest for unique ER ligands targeted to the vascular wall should lead to compounds that merge the pharmacological profiles of SERM and I-SERM agents. This review highlights the current bases for and approaches to selective ER modulation in the cardiovascular system. (Arterioscler Thromb Vasc Biol. 2006;26:2192-2199.)

Key Words: estrogen receptor ■ SERM ■ selective ER ligands ■ arterial wall ■ vascular protection

Coronary artery disease (CAD) accounts for one-third of all deaths in postmenopausal women. A potential role for estrogen in cardiovascular disease (CVD) protection has been long suggested by the observations that women have a reduced relative CVD risk as compared with men, but this benefit is lost after menopause, when circulating estrogen levels decrease dramatically. Since the cloning of estrogen receptors (ERs) and generation of ER knockout animals, several mediators and mechanisms have been identified attesting a beneficial role for estrogen on the cardiovascular system. When translated into the clinical setting, however, the value of observational and experimental studies has been disputed. In fact, the HERS (Heart and Estrogen/progestin Replacement Study) trial showed no benefit for hormone therapy with respect to cardiovascular events, and the estrogen/progestin arm of the Women’s Health Initiative study was terminated prematurely because of an increased risk of CVD events.

The disappointing results from clinical trials dimmed the optimistic perspective raised by preclinical studies. Yet, the body of evidence accrued by fundamental research in favor of beneficial cardiovascular effects of estrogen is too compelling to be ignored. To effectively translate this evidence from fundamental research into clinical practice, clearly there is a need to reappraise the molecular mechanism of ER activation and the specific physiological role of ER isoforms to identify more specific targets for pharmacological intervention. We focus here on the available molecular and pharmacological evidence supporting the opportunity to design agents combining ER-isoform and tissue selectivity targeted to the cardiovascular system, which may provide therapeutic benefits through direct vascular effects and control of vascular inflammation with limited adverse effects elsewhere.

Estrogen Receptors at Work

The biological actions of estrogen are largely mediated by 2 distinct ER isoforms, namely ERα and ERβ, that are widely distributed in tissues including the cardiovascular system (Figure 1A). The molecular pathways of ER activation have been deeply investigated. Following ligand binding, the ER undergoes conformational changes and biochemical modifications that induce release of inhibitory proteins (heat shock proteins), receptor dimerization, and interaction with DNA. In fact, nuclear ER acts as a transcription factor that modulates gene expression by directly binding to DNA at specific estrogen response elements (EREs) (Figure 1B). ER is also able to hinder transcription of promoters lacking EREs indirectly by interacting with nuclear transcription factors (Figure 1C). ER transcriptional activity may be regulated by intracellular signaling pathways even in the absence of estrogenic ligands (Figure 1D). For example, activation of growth factor receptors stimulates ER-mediated transcription. Ligand-independent ER activation has been recently demonstrated in vivo using transgenic reporter mice engineered to allow noninvasive imaging of ER transcriptional activity in all tissues of the alive mouse.
ERs regulate transcription of target genes in the nucleus also by recruiting coregulatory proteins such as coactivators. The distribution and relative abundance of coregulators promote the tissue-selective effects of drugs affecting ER function. By way of example, the steroid receptor coactivator-3 is highly expressed in vascular smooth muscle cells (SMCs) and appears to facilitate ER-dependent vasoprotective effects during vascular injury. The structural domains responsible for the interaction between ER and coregulatory proteins are scarcely homologous comparing ERα and ERβ, which explains the different ability to interact with coregulators and the diverse transcriptional responses evoked by these isoforms. In addition, ERβ may inhibit ERα action by forming heterodimers, indicating a “ying–yang” relationship between the 2 isoforms. Thus, the relative expression of ER isoforms drives the chain of cellular events mediated by ER activation.

As shown in Figure 1E, rapid nongenomic effects also follow ER–ligand interactions. These seem to be mediated by a receptor isoform, either distinct or identical to cloned ERs, localized at the plasma membrane (Figure 1B). Recently, an intracellular estrogen-binding site located on the endoplasmic reticulum has been described as a G protein–coupled receptor mediating intracellular calcium mobilization. Therefore, it appears that various ER forms expressed in vascular cells may be particularly efficient signal transducers and may use classical receptor domains for membrane targeting and insertion. The rapid regulation of nongenomic signal transduction pathways may ultimately influence the outcome of steroid-regulated gene expression, thus implying that rapid extranuclear and nuclear actions of ERs are coordinately integrated.

The multiple biomedical ramifications of ER-mediated cellular activation have been reviewed recently.

**Pharmacological Modulation of ERs**

Understanding how activated ERs elicit organ-specific effects is essential to design pharmacological agents reproducing estrogenic effects in target tissues without sharing untoward...
Because the endogenous ER agonist 17\beta-estradiol has the same binding affinity for ER\textsubscript{\alpha} and ER\textsubscript{\beta} in tissues,\textsuperscript{18} 2 approaches have been envisioned to accomplish selective modulation of ER activity: nonsteroidal tissue-selective ER modulators (SERMs) and selective ligands endowed with higher affinity for either ER isoform, which we here define as I-SERMs. The latter agents have been developed recently and are currently undergoing extensive evaluation (Table).

SERMs act by interacting with ER but differ from estrogens by eliciting agonist or antagonist effects depending on the target tissue.\textsuperscript{19,20} The most representative compounds of this class are tamoxifen and raloxifene (Figure 2). It is believed that selective ER modulation can be pursued because the conformational changes of ER induced by synthetic ligands may differ from those induced by 17\beta-estradiol.\textsuperscript{21,22} In particular, the interaction of agonists at the activating function (AF)-2 region of ER protein results in the recruitment of coactivators (Figure 1A), whereas antagonists favor recruitment of corepressors.\textsuperscript{20,22,23} Binding a SERM impairs AF-2 activity, whereas AF-1 is free to interact with other proteins, promoting the interaction of DNA-bound ER. Thus, based on available coregulators, the ER-bound SERM will act as a partial or even full agonist. In contrast, in cells lacking proteins interacting with AF-1, the SERM will prevent activation by estrogens and will therefore behave as a full antagonist.\textsuperscript{24} Yet, the molecular mechanisms underlying the tissue specificity of SERM effects are not completely elucidated.

Since the discovery of ER\textsubscript{\beta}, a major effort was made to develop compounds acting specifically on either ER subtype (Figure 2). The diarylpropionitrile DPN is a potent ER\textsubscript{\beta} agonist with a 30- to 70-fold selectivity over ER\textsubscript{\alpha}.\textsuperscript{25} Members of the triarylpyrazole class such as propylpyrazole trisphenol (PPT) are 400-fold more potent on ER\textsubscript{\alpha} than on ER\textsubscript{\beta}.\textsuperscript{26} By introducing basic side chain substituents such as those found in tamoxifen, ER\textsubscript{\alpha} selective antagonists have also been synthesized.\textsuperscript{27} I-SERMs induce specific ER conformations exposing interaction surfaces for coregulator recruitment quite different from 17\beta-estradiol.\textsuperscript{11} It is therefore conceivable that I-SERMs feature cell specificity of action, which should be investigated to develop more selective and specific pharmacological agents with estrogenic activity. Ideally, the major accomplishment of a quest for unique ER ligands would be the design of compounds (either ER agonists or antagonists or partial agonists) endowed with ER-subtype specificity and tissue- or even cell-specific action.

**Cardiovascular Action of Selective ER Ligands**

ER\textsubscript{\alpha} and ER\textsubscript{\beta} are widely distributed in the cardiovascular system.\textsuperscript{28,29} By regulating many genes involved in vascular biology, ERs exert direct effects on vascular cells and protect
from vascular injury.\textsuperscript{3,30} ER isoforms may have differential activity, as shown for plasminogen activator inhibitor (PAI)-1 promoter activity, which is increased by ER\(\alpha\) through an estrogen-dependent pathway and suppressed by ER\(\beta\) through an estrogen-independent mechanism.\textsuperscript{31} Yet, a systematic approach aimed at defining precisely the differences in vascular gene expression regulation by ER isoforms is lacking. On the other hand, the rapid vasorelaxant effect of estrogen mainly results from endothelial NO synthase (eNOS) activation on the plasma membrane of endothelial cells.\textsuperscript{32} This non-nuclear estrogen-signaling pathway involves direct interaction of ER\(\alpha\) with phosphatidylinositol 3-kinase.\textsuperscript{14} ER\(\beta\) activation in turn has been shown to rapidly enhance thrombin-induced aggregation in human platelets.\textsuperscript{33}

**Functional Role of ER Isoforms: Lessons From Animal Models of Targeted ER Deletion**

The generation of knockout (KO) mice lacking ER\(\alpha\) or ER\(\beta\) has provided insights into their specific role in the cardiovascular system.\textsuperscript{34} Overall, ER\(\alpha\) appears to mediate most cardioprotective actions of estrogen including nongenomic vasodilation. Estrogen treatment increases basal NO production in the aorta only in mice expressing functional ER\(\alpha\),\textsuperscript{35} and the vasorelaxation to 17\(\beta\)-estradiol becomes more pronounced in ER\(\beta\)-deficient mice.\textsuperscript{36} Because the NO-mediated vasodilator response to intraarterial 17\(\beta\)-estradiol in vivo is absent in double ER\(\alpha\) KO mice,\textsuperscript{37} ER\(\alpha\) appears to primarily modulate NO-mediated vasorelaxation, whereas ER\(\beta\) may sway ER\(\alpha\) activity. Estrogens also increase urinary excretion of cyclooxygenase (COX)-2–derived prostacyclin metabolites in ER\(\beta\) but not ER\(\alpha\) KO mice.\textsuperscript{38} An indirect interaction between ER\(\alpha\) and DNA via activator protein (AP)-2 has been shown to positively regulate COX-1.\textsuperscript{39}

In mice with genetic ER\(\alpha\) deletion, the cardioprotective role of estrogen in ischemia/reperfusion injury is lost\textsuperscript{40} and no detectable effects on vascular injury are observed after estrogen treatment.\textsuperscript{41,42} Of note, the protective mechanisms of estrogen treatment may vary with estrogen's genetic background.\textsuperscript{43} ER\(\alpha\) appears to directly modulate NO-mediated vasorelaxation, whereas ER\(\beta\) may sway ER\(\alpha\) activity. Estrogens also increase urinary excretion of cyclooxygenase (COX)-2–derived prostacyclin metabolites in ER\(\beta\) but not ER\(\alpha\) KO mice.\textsuperscript{38} An indirect interaction between ER\(\alpha\) and DNA via activator protein (AP)-2 has been shown to positively regulate COX-1.\textsuperscript{39}

Vascular Action of SERMs and I-SERMs

Whereas the vascular effects of endogenous ER ligands have been described in some detail,\textsuperscript{59} the use of established and novel selective ER ligands holds promise to evoke beneficial vascular responses with limited undesired effects in nonvascular tissues.

Raloxifene mediates acute ER- and endothelium-dependent vasorelaxation in rabbit coronary arteries.\textsuperscript{60} When chronically administered to ovariectomized rats, the drug stimulates eNOS expression\textsuperscript{61} and lowers ischemia susceptibility by increasing heart NO generation.\textsuperscript{62} Hypertension-induced endothelial dysfunction was significantly improved after raloxifene treatment through enhanced NOS activity and reduced reactive oxygen species production.\textsuperscript{63} Furthermore, endothelial cells exposed to serum from raloxifene-treated women show enhanced prostacyclin production.\textsuperscript{64} There is also evidence for an ER-independent vasodilating effect of raloxifene mediated by inhibition of L-type voltage-sensitive Ca\textsuperscript{2+} channels.\textsuperscript{65}

Similar to estrogen, raloxifene has antiproliferative properties in vascular SMCs, probably via interaction with ER\(\alpha\).\textsuperscript{66} In a model of rat carotid injury, raloxifene inhibits neointimal thickening\textsuperscript{67} and fosters reendothelialization.\textsuperscript{68} In addition, estrogen but not raloxifene reduces coronary artery plaque size in ovariectomized monkeys fed an atherogenic diet,\textsuperscript{69} indicating that the vascular effects of estrogen and raloxifene do not necessarily overlap. Thus, raloxifene may be a promising agent for treating some aspects of CVD, although its pharmacological effects are spread over multiple tissues. At present, SERMs with targeted action to the cardiovascular system are not available.

There are few studies with I-SERMs on cardiovascular function. Our group recently demonstrated that both ER\(\alpha\) and ER\(\beta\) are expressed in endothelial cells of rat aorta, but only selective ER\(\alpha\) agonists induce acute ER- and NO-dependent...
A role for ERα in improving endothelial dysfunction has been demonstrated in ovariectomized SHR rats treated with the selective ERα agonist Cpd1471.70 Accordingly, rabbits treated with the selective ERα agonist PPT are protected from ischemia/reperfusion injury, further supporting a beneficial role for ERα.71 The selective ERβ agonist DPN acutely relaxed precontracted mesenteric arteries from male rats,72 and the presence of ERβ apparently inhibits ERα-mediated NO relaxation,73 indicating that acute responses to selective ER agonists may vary dependent on the vessel used.74 Studies addressing cardiovascular function modulation by selective ER ligands in vivo are lacking to date.

Clinical Studies
Epidemiological evidence indicates potential cardioprotective effects of estrogens, whereas interventional studies do not.5,6,58 Evidence for beneficial effects of SERMs on CVD outcomes is so far limited.

The use of tamoxifen for the treatment of breast cancer during 5 years of follow-up was associated with a reduced risk of acute myocardial infarction in women,75 suggesting that tamoxifen protects against CAD.76 The MORE trial, which was designed to determine the effects of raloxifene on vertebral fractures in women with osteoporosis, showed that 4-year treatment with raloxifene does not affect CV risk in the overall cohort nor in any single year77 but does so in those women with increased CVD risk at baseline.78 However, a serious adverse event associated with raloxifene therapy is a 2-fold increase in the risk of venous thromboembolic events compared with placebo.79 The RUTH trial randomized 10 101 postmenopausal women (mean age, 67.5 years) with CAD or multiple risk factors for CAD to either 60 mg of raloxifene daily or placebo for a median of 5.6 years. The results show that raloxifene had no significant effect on the primary end point, coronary events, but it did significantly increase the risk of venous thromboembolism.80 Although the drug had no effect on stroke overall, there was a significant increase in death from stroke. Although reducing the risk of hormone-positive breast cancer and vertebral fractures, the benefits of raloxifene do not appear to outweigh the cardiovascular risks.

Antiinflammatory Action of ER Ligands
Inflammation develops concurrently with accumulation of minimally oxidized low-density lipoprotein in the arterial wall and is present at every stage of the atherosclerotic process. 17β-Estradiol is known to target several cell types that drive the inflammatory process. Early studies indicate that the hormone inhibits interleukin (IL)-1–mediated induction of endothelial cell adhesion molecules including membrane E-selectin and vascular cell adhesion molecule (VCAM)-1.81 We demonstrated that 17β-estradiol is able to block the synthesis of inducible NOS promoted by inflammatory stimuli in vascular82 and nonvascular tissues.83 The early vascular injury response as a whole is attenuated by 17β-estradiol, as shown by the reduced expression of adhesion molecules, chemokines and proinflammatory cytokines in rat carotid arteries, which in turn reduce leukocyte recruitment and chemiotaxis.84 Recently, the molecular players involved in estrogen action have been investigated, pointing to the relevant role of ERα. This receptor isoform inhibits inflammatory gene transcription; our recent work linked this effect with a unique mechanism of action among antiinflammatory drugs involving inhibition of nuclear factor (NF)-κB nuclear translocation.85 The relevance of the NF-κB pathway as a target of estrogen anti-inflammatory activity is further underlined by the identification of pathway-selective ER ligands, such as WAY-169916, which retain the ability to antagonize the NF-κB pathway while being devoid of classical estrogenic action on reproductive tissues (Table).86 Interestingly, WAY-169916 protects against cardiac ischemia/reperfusion injury, although displaying different activity on cardiovascular end points as compared with 17β-estradiol.87 Accordingly, evidence has accumulated as to the role of ERα in preventing inflammatory-related cerebrovascular and neural disease using ER-knockout mice and I-SERMs.43,88–90 On the other hand, although ERβ-selective ligands reduced clinical signs of inflammatory bowel disease and rheumatoid arthritis,91 the specific role of ERβ in inflammation is not yet clarified. Of note, estrogen anti-inflammatory effects may rely on vascular health because ER expression is regulated by oxidative stress.92,93 Accordingly, the expression pattern of ER isoforms is altered in vascular SMC from streptozotocin–diabetic as compared with normoglycemic rats.49

Thus, experimental evidence accumulated so far indicates that additional benefits of SERMs and I-SERMs may be anticipated if these compounds shared the antiinflammatory activity of 17β-estradiol on the innate immune system.

In humans, hormone therapy has a significant impact on levels of inflammatory biomarkers.94 Yet, an important issue in this regard is the route of administration, because transdermal as opposed to oral estradiol administration does not elevate C-reactive protein (CRP).94 Raloxifene does not affect, whereas tamoxifen markedly reduces, plasma CRP95 and fibrinogen96 levels, probably by direct ER antagonism. Although lowering inflammatory markers such as CRP appears to decrease cardiovascular risk, it is unclear whether selective ER ligands may affect clinical end points through such effects.97

Conclusions and Open Questions
Currently, ERα appears to be a primary target for both cardiovascular and inflammatory disease. We still have to address the pharmacological limitations of poor ER-subtype and tissue selectivity of physiological estrogens. A step forward should come from basic research to better understand how SERMs and I-SERMs work at the tissue level and how ER function is altered under pathophysiological conditions. An emerging determinant of therapeutic efficacy for ER ligands is the cyclic or permanent change of ER expression patterns in the vessel wall before and after menopause.56 Accordingly, clinical outcomes may be affected by vascular health in individual patients, suggesting the importance of treatment timing.98 So far, compounds fulfilling both criteria of ER-subtype and tissue selectivity are lacking (Table). Although the quest for such molecules still requires solid
fundamental knowledge of ER molecular pharmacology as well as extensive clinical testing of efficacy and safety, this strategy represents the way ahead to exploit the benefits of estrogenic compounds on the vascular wall while, at the same time, limiting undue effects elsewhere.

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