Gender Differences of Cardiovascular Disease
New Perspectives for Estrogen Receptor Signaling

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During the last century, atherosclerotic cardiovascular disease has burgeoned from a relatively minor disease worldwide to a leading cause of morbidity and mortality.1 Still, atherosclerosis is rare in premenopausal women except in cases with known positive family history of coronary artery disease (CAD) or genetic abnormalities of lipid metabolism.2 The prevalence of CAD in men is several times higher than that of age-matched premenopausal women, but these gender-based differences narrow after menopause, when the protection against vascular disease is gradually lost (Figure 1).2 Indeed, the risk of atherosclerosis is increased when estrogen production stops, either naturally or after surgery3 or in woman with impaired ovarian function.4 The time since menopause is a major risk factor for the development and progression of atherosclerotic lesions, as well as the development of hypertension.5 In fact, cardiovascular disease has claimed the lives of more females than males in every year since 1984, although women develop CAD ∼10 years later than men.6 In view of these epidemiological data, estrogens have been implicated in the primary prevention of atherosclerosis in premenopausal women.

Experimental studies have shown that natural estrogens, such as 17β-estradiol, protect blood vessels from atherosclerotic lesion formation,7,8 lower plasma levels of low-density lipoprotein cholesterol and lipoprotein Lp(a), and raise plasma levels of high-density lipoprotein cholesterol.8,9 17β-Estradiol also accelerates endothelial cell recovery after balloon injury10 and inhibits vascular smooth muscle cell (VSMC) proliferation.11,12 Moreover, the phenolic ring structure provides strong antioxidant activity of 17β-estradiol.13 In contrast to these observations, large randomized clinical trials in postmenopausal women with cardiovascular risk factors or CAD, both using conjugated equine estrogens and medroxyprogesterone acetate as hormone “replacement,”14 have recently questioned these atheroprotective effects, because the results showed no effects or even an increase in cardiovascular morbidity and mortality, such as thrombosis or stroke.15,16 These divergent findings resulted in confusion about whether a substitution therapy with natural or novel synthetic sex steroids could represent a therapeutic option for the treatment of atherosclerosis and its complications and have even led to new guidelines regarding hormone replacement therapy.17 One key to solving such an important public health issue would be to better understand the complex mechanisms of acute and chronically administered estrogen action in the vasculature.

A Role for Estrogens in Vascular Homeostasis in Females and Males

It has been reported recently that estrogens are not only formed in the female and male reproductive tract but also locally in the vasculature after conversion of testosterone catalyzed by the enzyme aromatase.18,19 Interestingly, the lack of aromatase in humans is associated with progressive virilization, eunuchoid habitus, osteopenia, and abnormal lipid profiles.20 It is, thus, not surprising that inhibition of aromatase increases atherosclerotic lesion formation in male mice.21 In addition, impaired endothelium-dependent vasodilation has been demonstrated recently in healthy men after pharmacological inhibition of aromatase.22 These findings strongly suggest the possibility that physiological effects of 17β-estradiol are also present in the male vasculature and more important than previously appreciated.

The diverse effects of estrogens on the vasculature are the result of a multitude of actions on various components of the vascular wall, such as endothelial and smooth muscle cells, and implicate a complex interplay of transcriptional, as well as nontranscriptional pathways.23 The mechanisms responsible for many genomic effects involve binding of estrogens to the nuclear estrogen receptors (ERs), which exist in 2 different forms, ERα24 and ERβ.25–27 ERα and ERβ have been detected in human endothelial and VSMCs,28,29 and their expression is altered in human atherosclerosis.30 Both ERα and ERβ mediate physiologically important effects in the vasculature. Premature CAD has been reported in a 31-year-old man with a disruptive mutation in the ERα gene.31 In addition, the repair process of atherosclerotic lesions in previously healthy vessels, as well as estrogen-mediated effects on the lipid profile, are dependent on the presence of a functional ERα in both female32 and male33 mice. In carotid arteries of healthy female mice, the protective effects of estrogen in response to vascular injury are mediated by ERα.34 Surprisingly, male ERβ-deficient mice develop sustained systolic and diastolic hypertension as they age,35 and polymorphisms in the ERβ gene have been associated with the development
of hypertension in postmenopausal women.\textsuperscript{36} ER\textbeta mRNA expression has also been shown to be upregulated after vascular balloon injury in males,\textsuperscript{37} and remodeling of veins of the vasculature of men.\textsuperscript{38} These observations suggest that targets of “female” sex hormones are also of physiological importance in the vasculature of men. Thus, not only the female but also the male cardiovascular system seems to be an important source and target for estrogens. Nevertheless, there is doubt that treatment of male CAD patients with estrogen receptor–activating compounds, as shown experimentally in mice\textsuperscript{39} and previously unsuccessfully attempted in patients,\textsuperscript{40–41} represents a treatment option to interfere with disease progression in patients with coronary atherosclerosis.

**Regulation of Nuclear ER Expression and Function**

Cellular responses to estrogens are, in large part, controlled by expression of nuclear ERs, because the saturation of the cellular capacity to mediate an estrogen response occurs at ER titers well above those encountered under physiological conditions.\textsuperscript{42} The role of the ER as a limiting factor seems even more important, because ER expression in the vasculature is highly regulated, similar to that in other estrogen-sensitive tissues. For example, 17\beta-estradiol downregulates ER\textalpha expression in the vena cava of ovariectomized rats.\textsuperscript{43} In contrast, ER\textalpha expression in the thoracic and abdominal aorta of ovariectomized rats is unaffected by chronic 17\beta-estradiol treatment.\textsuperscript{44} In rat cerebral arteries, ER\textalpha gene expression decreases after ovariectomy and increases after 17\beta-estradiol replacement therapy.\textsuperscript{45} Moreover, deprivation of 17\beta-estradiol in rats is associated with a significant decrease in the vascular expression of ER\textbeta,\textsuperscript{46} and whereas 17\beta-estradiol replacement upregulates ER\textalpha, it does not affect ER\textbeta gene expression.\textsuperscript{46} Taken together, these observations suggest that the expression of ERs is highly regulated and varies considerably among different types of blood vessels.

Two major mechanisms have been identified that regulate ER expression. First, the autologous downregulation pathway (Figure 2A) involves the interaction of an activated ER with its own gene sequence and subsequent suppression of its transcription, similar to the mechanism by which an ER regulates expression of any other target gene.\textsuperscript{47,48} Through this mechanism, estrogens inhibit ER expression at the mRNA level. The second mechanism, the ubiquitin-proteasome proteolysis pathway (Figure 2B), is involved in the rapid degradation of various proteins. An enzymatic cascade leads to activation of ubiquitin, a highly conserved small protein, which marks a protein for subsequent degradation by the 26S proteasome.\textsuperscript{49–51} Thus, this mechanism downregulates the ER protein concentration without altering ER mRNA expression. Both the autologous downregulation and the ubiquitin-proteasome proteolysis pathways differently regulate expression of ERs in vascular endothelial and smooth muscle cells. In human aortic smooth muscle cells, expression of ER\textalpha is controlled by the autologous downregulation pathway, whereas the expression of ER\textbeta is governed by the ubiquitin-proteasome proteolysis pathway.\textsuperscript{52} In contrast, in human endothelial cells, expression of both ER\textalpha and ER\textbeta is regulated by proteasome-mediated degradation pathways.\textsuperscript{53}

In addition, epigenetic regulation by methylation of ER gene represents an important mechanism by which cells modulate ER gene expression (Figure 2C).\textsuperscript{54} In the cardiovascular system, downregulation of ER\textalpha has been attributed to ER\textalpha gene methylation, which occurs more frequently with increasing age in the human atrial myocardium.\textsuperscript{55} Moreover, methylation of the ER\textalpha gene has been demonstrated in atherosclerotic plaques.\textsuperscript{55} Notably, estrogen-mediated activation of endothelial NO synthase (eNOS) is mediated by ER\textalpha, which leads to enhanced synthesis of NO, thus promoting vasodilation and inhibiting inflammation.\textsuperscript{56} Therefore, methylation-associated inactivation of the ER\textalpha gene may play a role in atherogenesis. In addition, it is known that ER expression levels may be regulated by selected hormonal signals and growth factors.\textsuperscript{57} For example, ER expression has been shown to be downregulated by progestins (Figure 2D).\textsuperscript{57} Taken together, vascular ER expression is controlled by numerous complex mechanisms, which are likely to affect vascular functional efficacy as well as adverse effects of estrogens, which may have relevant pharmacological and therapeutic consequences.

**Classical Molecular Pathway of Estrogen Action**

Estrogen molecules have classically been thought to diffuse into the cell and bind to nuclear ERs, and this way of action has been referred to as the classical or ligand-dependent mechanism (Figure 3A). As members of the large superfamily of nuclear receptors, ERs function as ligand-activated transcription factors.\textsuperscript{58,59} Once an estrogen ligand binds to an ER, a conformational change occurs in the protein structure, which triggers the dissociation from the 90-kDa heat shock protein\textsuperscript{60,61} to allow the dimerization of the receptor.\textsuperscript{59} Subsequently, the receptor dimers interact with other transcriptional cofactors with the estrogen response element (ERE), a specific regulatory DNA sequence present in the promoter region of target genes.\textsuperscript{59} ER\textalpha and ER\textbeta interact with their ERE as a homodimer in cells expressing only 1 ER subtype or possibly via the formation of heterodimers in cells expressing both receptor subtypes,\textsuperscript{62} which may affect the efficiency of receptor function as demonstrated for other steroid receptors (Figure 4).\textsuperscript{63} In addition, a second nonclassical mechanism involves the interaction of estrogen-bound nuclear ER with transcription factors, such as activator protein-1 or Sp1.\textsuperscript{64} Thus, this mechanism is independent of EREs and
involves no direct contact of ERs with DNA (Figure 3B). Activator protein-1 and Sp1 have been shown to bind to ERs and interact with their cognate binding sites in the promoter region of target genes.65,66 Therefore, both signaling pathways result in changes in gene expression that induce an overall physiological response occurring within hours after estrogen exposure. Naturally, these processes do not involve second messenger signaling pathways.

Novel Mechanisms of Rapid Estrogen Signaling: Identification of a G-Protein–Coupled Membrane–Bound ER

Estrogen signaling in the human vasculature is more complex than previously recognized, because many estrogen-regulated effects are too rapid to be mediated by transcription and protein synthesis and, thus, cannot be explained by the classical mode of action via nuclear receptors. More than 30 years ago, Pietras and Szego67,68 reported that estrogen binding sites in plasma membranes were coupled to the rapid activation of intracellular signaling pathways. It was subsequently demonstrated that physiological levels of estrogen exert direct vasodilating effects in vitro within minutes, which are independent of an intact endothelium (Figure 5).69 Moreover, 17β-estradiol has been shown to cause vasodilation by activation of eNOS through the phosphatidylinositol 3-kinase–Akt pathway.70–72 Thus, new models of estrogen action began to evolve, and it has been suggested that these rapid effects are nongenomic and involve membrane-mediated estrogen signaling (Figure 3C).73,74 It was first proposed that a subpopulation of ERα is localized to endothelial cell caveolae where they are coupled to eNOS in a functional signaling module promoting the release of NO.56 In addition, transfection studies suggested that ERβ also localizes to the cell membrane,75 and the presence of plasma membrane–associated estrogen-binding proteins without structural similarity to nuclear ERs has been reported.76 Membrane-bound ERs (mER) modulate cell membrane ion channels,77,78 G protein–coupled receptors,79 tyrosine kinases, and mitogen-activated protein kinases80 and are able to activate adenyl cyclase81 and phospholipase C.82 Interestingly, not only rapid effects induced by estrogen seem to be mediated by mER, because the inhibitory effects of estrogens on DNA synthesis

Figure 2. Mechanisms regulating ER expression. The autologous downregulation pathway (A) involves the interaction of a liganded ER to an ER gene, leading to suppression of its own transcription. In the ubiquitin–proteasome proteolysis pathway (B), an ER protein gets marked by ubiquitin (Ub) and subsequently degraded by the 26S proteasome. This mechanism downregulates ER protein concentrations without alterations in mRNA expression. ER expression is further modulated by epigenetic methylation (C), because the methyltransferase DNMT1 transfers methyl groups to the ER gene, thereby inhibiting transcription. Moreover, growth factors and hormonal signals, such as progesterone, are involved in regulation of ER transcription (D) (adapted in part from References 51, 54).
in VSMCs, but not in endothelial cells, apparently do not require entry of 17β-estradiol into the cell. However, the precise subtype(s) of mER affecting VSMC function has not yet been elucidated, but this example demonstrates that mER signaling through kinase cascades also impacts transcription (Figure 3D). Signaling from mER to the nucleus has also been shown in breast cancer cells, where mER trans-activates the epidermal growth factor receptor (Figure 3E), resulting in activation of downstream signaling and phosphorylation of the endogenous nuclear ER, thus upregulating its transcriptional activity. This mER has been identified as a G protein–coupled receptor, termed GPR30. Two groups recently rendered the properties of GPR30 more precisely to be a protein structurally unrelated to the nuclear ER but with typical binding characteristics of an ER. Unlike trans-activation of the epidermal growth factor receptor, ligand binding to GPR30 initiates signaling cascades by activation of a stimulatory G protein and upregulation of adenylyl cyclase with subsequent formation of cAMP, and 17β-estradiol is able to trigger this rapid intracellular response, whereas estrone and estriol are inactive. Interestingly, increases of cAMP after short-term exposure to 17β-estradiol have also been observed previously in human coronary arteries (Figure 5). In view of these recent findings, different concepts have diverged whether a subpopulation of ERα and ERβ, GPR30, a yet undefined receptor, or a combination represents the putative mER. Moreover, reports on the cellular localization of GPR30 differ, suggesting that GPR30 could either be located at the plasma membrane or within the endoplasmatic reticulum.

There is evidence to suggest that GPR30-mediated estrogen signaling occurs in the vasculature, because GPR30 is widely distributed in various tissues, including the human heart, aorta, and umbilical vein endothelial cells. GPR30 mRNA is upregulated by vascular shear stress in humans and, thus, may be involved in shear stress–mediated regulation of endothelial cell functions. In contrast, GPR30 was not detected in sheep endothelial cells of the aorta and pulmonary artery. Preliminary results from our laboratory suggest a role for GPR30 in rapid vascular estrogen signaling, because we detected GPR30 mRNA in both human internal mammary arteries and saphenous veins. Gene expression of

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**Figure 3.** The multifaceted mechanisms of estrogen signaling. The classical mechanism (A) involves binding of an activated ER to an ERE leading to upregulation or downregulation of target gene transcription. In addition, an estrogen-bound ER can interact with transcription factors, such as activator protein-1 and SP-1, and, thus, affects gene expression independent of EREs via activator protein-1/SP-1-binding sites (B). Estrogen also binds to membrane-associated estrogen-binding proteins (ER subpopulation, GPR30, or a yet undefined receptor). This pathway involves intracellular signaling cascades and either triggers rapid nongenomic cellular responses (C) or results in changes in transcriptional activity (D). The mER may also cross-talk to the trans-activation of growth factor receptors (GFR; E), leading to phosphorylation of ERs through intracellular signaling cascades, thereby modulating target gene transcription. In response to growth factors (F), the nuclear ERs can further be activated independently of an estrogen ligand by similar mechanisms (adapted in part from References 64, 88).
GPR30 was sensitive to regulation by 17β-estradiol in the artery but not in veins (M.R. Meyer, E. Haas, M. Barton, unpublished data, 2006). Interestingly, it has also been demonstrated that antagonists thought to be specific for nuclear ERs, such as ICI 182 780 and tamoxifen, also bind to GPR30 and have opposite actions on this alternative mER-mediated pathway, acting as estrogen agonists on GPR30 and activating G proteins.\textsuperscript{86,87} These newly discovered properties of widely used drugs might have profound implications for treatment strategies with estrogens and also antiestrogens.\textsuperscript{88} Still, the design of drugs that could selectively activate or repress an mER would help to identify the contribution of this receptor pool to the overall response to estrogens and may provide possible new targets for therapeutic interventions in hormone-sensitive diseases, such as cancer or atherosclerosis.

**Vascular Protection: Which Role Do Estrogens Play?**

Despite the documented role of endogenous estrogens for primary prevention in premenopausal women, the lack of information on the high and complex regulation of vascular ERs and the multifaceted mechanisms of estrogen signaling currently questions a defined role of exogenous estrogens for cardiovascular therapy. In particular, various aspects of estrogen action are not yet fully understood. By these means and in view of the present data available on postmenopausal hormone “substitution” therapy with questionable compounds given at very high concentrations,\textsuperscript{5,14,93} the most important goal remains the prevention of atherosclerosis, the leading cause of death in women. An early and exact evaluation and optimization of the cardiovascular risk factor profile, such as cessation of smoking, normalization of body weight and blood pressure, regular physical activity, and even statin treatment in patients in whom CAD has been diagnosed, remain essential treatment requirements.

Because of the increased incidence of cardiovascular complications, conjugated equine estrogens and, particularly, medroxyprogesterone acetate are no longer suitable for postmenopausal hormone therapy. However, it remains to be
shown whether a substitution therapy with natural or novel synthetic steroids could represent a therapeutic option for the treatment of atherosclerosis and its complications, particularly in younger postmenopausal women. In addition to composition, dosage, and application form of a hormone preparation, the timing of the initiation of a therapy, the status of the patient’s cardiovascular health, and the duration of treatment, as well as pharmacological interactions with other drugs, play important roles. Moreover, it remains to be demonstrated whether current regimens of postmenopausal hormone therapy should be revised toward those resembling closer physiological cycling in 17β-estradiol plasma levels to gain a better therapeutic effect.

Certain nonsteroidal drugs can interact with ERs and possibly offer additional therapeutic options. It has been demonstrated recently that nebivolol, a novel β1-adrenoreceptor blocking agent, has striking structural and chemical similarities to 17β-estradiol. Thus, it is not surprising that nebivolol has specific endothelium-dependent vasodilating properties, because it interacts with the endothelial ER and induces NO-mediated vasodilation via activation of eNOS. Indeed, increased vasodilator activity has been observed after oral administration of nebivolol in humans. These findings suggest that this drug could increase NO bioactivity in the vessel wall and, hence, may reduce atherogenesis and thrombosis. In fact, the results of the Study of the Effects of Nebivolol Intervention on Outcomes and Rehospitalization in Seniors with heart failure (SENIORS) trial demonstrated that nebivolol treatment of elderly patients with heart failure reduces mortality and morbidity and is well tolerated. Interestingly, the therapeutic benefit seems to be greater in women. It remains to be demonstrated, however, whether an interaction of nebivolol with ERs also exists in vivo in male and female patients and whether there are additional effects that interfere with the progression of atherosclerotic vascular disease.

In summary, the highly complex regulation of vascular ER expression and the identification of GPR30 as a novel mER have shed a new light on estrogen signaling and action. A cellular response to estrogen implicates a high plasticity, involving the activation of ERs in different cellular locations, and triggering various signaling cascades and/or transcription of genes in response to the same agonist. This activation results in both rapid and sustained effects, but many of the underlying mechanisms are still poorly understood. Thus, further research is needed to better assess the potential benefit and the adverse effects of future estrogen-based vascular therapies.

Acknowledgments

Work by the authors is supported by the Swiss National Foundation (SCORE 3200-058426.99, 3232-058421.99, and 3200-108258/1) and the Hanne Liebemann Stiftung Zürich.

References

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Hypertension. 2006;47:1019-1026; originally published online May 1, 2006;
doi: 10.1161/01.HYP.0000223064.62762.0b
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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