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Nongenomic, Estrogen Receptor–Mediated Activation of Endothelial Nitric Oxide Synthase

How Does It Work? What Does It Mean?

Michael E. Mendelsohn

> The administration of estrogens in animals tends to inhibit the development of atherosclerosis... [Therefore], attempts are being made in human males to prevent further progress of the disease in cases of angina or previous coronary thrombosis by long-range estrogen therapy. This work is in its infancy. It is hoped that, with further research, compounds might be found that eliminate the undesirable action of such hormones and yet retain its beneficial effects on arteries (Samuel A. Levine, 1958).[1]

In the past decade, a great deal of attention has been focused on whether administration of estrogens can prevent cardiovascular diseases in postmenopausal women. Despite a wealth of supportive observational data,[2] two recent clinical trials, the HERS trial[3] and the ERA trial,[4] have called into question whether current hormone replacement therapy (HRT) regimens have any cardioprotective effects. However, these studies only support that women with known coronary artery disease who are many years past menopause should not be started on HRT for secondary prevention of heart disease. The therapy of choice in such patients is well accepted to be direct risk factor modification, including antihypertensive therapy, treatment of diabetes, and an HMG-CoA reductase inhibitor, as appropriate, as well as lifestyle changes. Furthermore, neither HERS nor ERA addresses primary prevention against cardiovascular disease by HRT. Thus, the potential role of estrogens in protecting against cardiovascular diseases remains controversial and unresolved.[5] The protective effects of estrogen for cardiovascular diseases are supported by many basic science studies demonstrating that estrogen affects both systemic factors important to the pathophysiology of cardiovascular disease and also has direct effects on the heart and vasculature (reviewed in Reference 6). The effects of estrogens are mediated by estrogen receptors. To date, only two estrogen receptors have been identified, ERα and ERβ, and both of these receptors are expressed and functional in cardiovascular tissues, supporting a direct role for estrogen in cardiovascular physiology. Estrogen receptors, like all members of the nuclear hormone receptor superfamily, are ligand-activated transcription factors. Estrogen receptors in vascular cells can alter the expression of a number of target genes, and this mediates the direct, long-term (genomic) effects of estrogen on vascular tissues.[6]

More recently, a second mechanism for a direct effect of estrogen on the vasculature has been identified. Animal and human studies have shown that physiological levels of estrogen can rapidly cause vasodilatation (reviewed in Reference 6). Studies in human subjects support that this effect is largely mediated by activation of endothelial nitric oxide synthase (eNOS), and recent studies of cultured endothelial cells further support this conclusion. In 1997, two laboratories[7,8] reported that short-term exposure of cultured endothelial cells to estrogen activates eNOS, causing increased release of NO. These investigators showed further that this rapid effect of E_2 could be inhibited by ICI 182,780, a relatively specific inhibitor of the two known nuclear hormone receptors for estrogen. Thus, these initial data suggested a novel, nontranscriptional action of the ER protein. This surprising and seminal observation has led to a series of studies establishing a role for ERα in the rapid activation of eNOS. A subsequent report demonstrated that indeed ERα mediates the short-term effects of estrogen on eNOS activity and that the acute response of eNOS to E_2 can be reconstituted in cells lacking ERα and eNOS by cotransfection of cDNAs for these two proteins.[9]

Three new studies now shed significant light on the signal transduction pathway that mediates the rapid, nongenomic activation of eNOS by estrogen and ERα and the intracellular localization of the molecules involved in this pathway. In a recent issue of *Circulation Research*, Haynes et al[10] provided evidence that the activation of eNOS in endothelial cells by ERα involves the PI3-kinase-Akt pathway. In studies of eNOS activation in the human endothelial cell hybridoma line EA.hy926, these investigators demonstrate inhibition of estrogen-induced NO_2^- release by a pharmacological inhibitor of PI3-kinase and show that 17β-estradiol (E_2) and E_2-BSA increase the phosphorylation of both Akt and eNOS. These investigators further show using adenoviral infection approaches that a dominant-negative Akt inhibits NO_2^- release from cells stimulated by either E_2 or 17β-estradiol conjugated to bovine serum albumin (E_2-BSA). These data suggest that the phosphatidylinositol 3-kinase (PI3-kinase)-Akt-eNOS activation pathway, identified simultaneously by two laborato-

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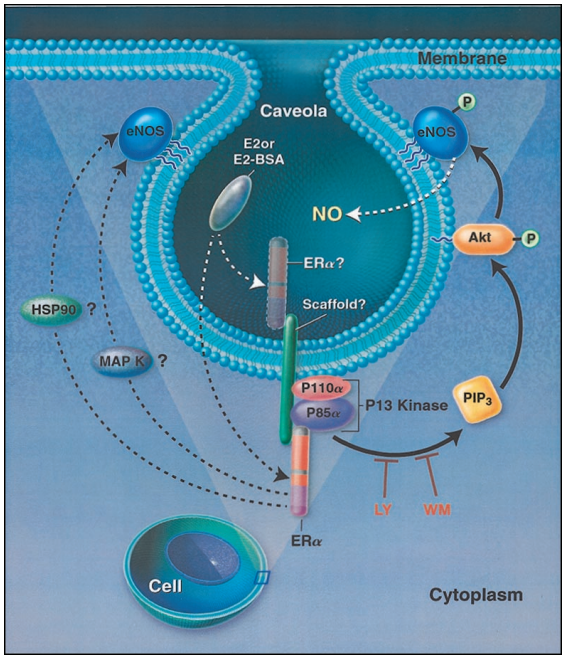
ries last year,[12,12a] is recruited by estrogen in some fashion. There are several caveats to this interesting study. Akt kinase activation is never directly demonstrated, and the role of ER itself is not systematically examined with either pharmacological or molecular approaches. Although the authors show in separate experiments that E_2 leads to phosphorylation of Akt, phosphorylation of eNOS, and increased NO_2^- production, they do not show that either PI3-kinase inhibition or dominant-negative Akt adenovirus prevent E_2-induced Akt or eNOS phosphorylation. In addition, the use of the HUVEC-sarcoma fusion cell line raises interesting questions. These cells express a truncated 46-kDa form of ERα that is recognized immunologically by antibodies to the carboxyl terminus of ERα,[10] but the ER in these cells has not yet been cloned or characterized. What domains are actually present and responsible for eNOS activation in these EA.hy926 cells remains unclear, as does whether this shorter ER is present in "normal" endothelial cells. Also, although apparently not expressed with the conditions in this study,[10] EA.hy926 cells are capable of expressing the 66-kDa ER under certain culture conditions (M. Mendelsohn, unpublished results, 2000). Finally, although these authors argue that the effects they observe are mediated by a "membrane receptor," these conclusions are based on the use of E_2-BSA as a membrane impermeant, cell surface–specific ER agonist. However, it is not possible using E_2-BSA to conclude that any ER engaged is truly in the membrane. It is now recognized that E_2-BSA has free E_2 associated with it and is capable of activating transcription, but if the associated free E_2 is removed by dialysis, E_2-BSA does not itself bind to ER.[13] Reports of membrane ERs in a variety of cells have existed for more than 20 years[14] (reviewed in Watson[15] and Watson and Gametchu[16]). However, to date, no one has clarified the precise location of the putative membrane ER and many studies have relied heavily on E_2-BSA. This highlights one of the major limitations in this field at present: the lack of a truly cell-impermeant ER agonist.

A study that appeared in the September 28 issue of *Nature* from the Liao laboratory[17] also implicates Akt in the activation of eNOS by estrogen and provides substantial additional insight into the signal transduction pathway involved in ERα-eNOS activation. These investigators demonstrate for the first time a direct interaction of ERα with the p85 regulatory subunit of PI3-kinase. In addition, extensive experiments are provided that show inhibition of eNOS activation by wortmannin in intact endothelial cells, direct estrogen stimulation of ERα-associated PI3-kinase activity over a 15- to 20-minute time course, and coimmunoprecipitation of ERα and p85, with simultaneous generation of the PI3-kinase product, PIP3. Interestingly, the time course of PI3-kinase activation by estrogen is somewhat slower than activation of PI3-kinase by growth factors such as insulin and is similar to previous reports of mitogen-activated protein (MAP) kinase activation by estrogen-ERα.[9] One particularly intriguing finding in this study is the identification of a direct, E_2-dependent interaction of ERα and p85 in in vitro studies using only recombinant human ERα and GST-p85 fusion proteins. Although this demonstrates a direct interaction between these two proteins, it was not shown whether this binding event also leads to activation of PI3-kinase, leaving open the possibility that other proteins also are required to activate the PI3-kinase complex. These investigators note further that the heat shock protein 90 (HSP90), reported previously to bind to and facilitate eNOS activation,[18] actually disrupts the interaction of ERα and p85. In supplementary data provided with the manuscript, the authors also show that ERβ does not interact directly with p85.[17]

In a study[19] in this issue of *Circulation Research*, data are presented localizing the functional ERα-eNOS signaling complex to endothelial cell caveolae, the membrane microdomains well-known to be enriched in many signaling molecules, including eNOS.[20–22] These investigators provide immunological evidence that ERα is expressed in caveolae using both amino- and carboxy-terminal domain ER antibodies. They show further, in highly purified caveolae preparations, that estrogen exposure leads to rapid and robust activation of eNOS that is fully inhibited by the ER antagonist ICI 182,780. These investigators also provide evidence that this signaling event in isolated caveolae is somehow calcium-dependent, suggesting that a small amount of local calcium may be important for ERα activation of eNOS in caveolae. These data, along with several recent studies,[23,24] may be relevant to the controversy in the literature as to whether estrogen activation of eNOS is calcium-dependent or calcium-independent. Importantly, the Shaul laboratory's demonstration of an intact, fully functional ERα-eNOS signaling system in caveolae identifies purified caveolae as an excellent model system in which to dissect this pathway further. Taken together, these three studies allow construction of a model of this interesting new signal transduction pathway (Figure) that now can be tested and further refined.

Four central issues remain to be clarified at present in this rapidly evolving area of research: (1) How, if at all, does the PI3-kinase-Akt pathway interact with other signaling molecules previously implicated in ER-eNOS signaling, such as MAP kinase and HSP90?; (2) Which ERs and ER domains can mediate rapid eNOS activation and which actually do so in vivo?; (3) Where and how is the ER localized in caveolae?; and (4) what, if any, is the physiological relevance of these rapid activation pathways?

Several laboratories have shown that E_2 rapidly activates MAP kinase in endothelial cells,[9,25] as it does in other cells and tissues,[26,27] but the role of estrogen-ER–mediated activation of MAP kinase in eNOS activation remains unclear (Figure). It has been shown previously in intact endothelial cells that pharmacological inhibition of MAP kinase with PD98059 prevents activation of eNOS by estrogen,[9] whereas the recent report regarding ER and PI3-kinase finds that this same inhibitor does not alter activation of eNOS by estrogen.[17] This may be due to differences in the dose of the inhibitor used or in the cell types studied, but the central question remains: does MAP kinase activation play any role in the activation of eNOS? Why are both the MAP kinase and PI3-kinase signaling pathways rapidly recruited by estrogen-ER? Is MAP kinase downstream of PI3-kinase in this pathway? The role of ERβ also needs further exploration. Although in the recent report regarding a p85-ERα interaction no interac-



Composite model of nongenomic, ER-mediated activation of eNOS, based on published data and the three recent studies discussed in this editorial.[10,17,19] These recent studies show immunological localization of ERα to caveolae, direct interaction of ERα with the p85 subunit of PI3-kinase, and activation of the PI3-kinase-Akt-eNOS pathway. However, many issues remain unresolved, in particular, including whether the ERα hormone-binding domain is actually inside or outside the membrane, how this ERα is tethered to the caveolae, and the precise primary sequence of this membrane-associated ER. The specific domains of ERα that are important for the p85 interaction, as well as the identity of other potential proteins that participate in the proximal signaling events in this pathway, also remain unknown. Most importantly, compelling evidence is lacking that this pathway for nongenomic activation of eNOS by estrogen has a physiological or cardioprotective role. For details, see text. Scaffold indicates protein tethering ER to the membrane, such as a known transmembrane receptor; LY, LY294002; and WT, wortmannin (PI3-kinase inhibitors).

tion between GST-p85 and ERβ is observed,[17] recent whole-cell studies support that ERβ may also be capable of activating eNOS in a rapid fashion (P. Shaul, personal communication, February 2000). Assuming both of these observations prove correct, this suggests that ERα- and ERβ-mediated activation of eNOS may proceed through entirely different pathways. The relative importance of these two pathways in eNOS activation is unclear and would be expected to vary, depending on the level of expression of the two ERs in a given cell type. Does ERα use the PI3-kinase pathway preferentially, whereas ERβ recruits mainly MAP kinase? The kinetics of estrogen-mediated eNOS activation also remain somewhat unclear, given that they appear to differ between laboratories, and this may be related to the existence of several signal transduction pathways. For example, Haynes et al[10] detect estrogen-mediated phosphorylation of Akt within 5 minutes. Simoncini et al,[17] in contrast, do not detect Akt activation until 15 to 20 minutes after estrogen stimulation of endothelial cells and also do not detect PIP3 generated any earlier than 15 minutes after E_2 stimulation.

The role of HSP90 in estrogen-mediated activation of eNOS also is confusing at present. Although one laboratory reports that HSP90 can disrupt the ERα-p85 interaction,[17] another has previously reported that HSP90 directly activates eNOS.[18] A third group has shown that estrogen actually promotes the association of HSP90 and eNOS as a potential mechanism of rapid activation of eNOS by E_2.[28] The relationship between these somewhat contradictory observations remains unclear. Furthermore, although immunological data exist to support that the nuclear hormone receptor ERα is an in vivo membrane-associated ER, direct isolation and protein sequencing of ER from purified caveolae are required to formally prove that full-length ERα is indeed the in vivo membrane-associated form of the receptor. It is also important to consider whether other proteins participate in a complex with ER and p85. The structures of ERα and ERβ have now been solved, and based on these structures, and their primary sequence, it is clear that neither is a transmembrane protein itself. Thus, the putative membrane ER, which is discussed so widely in the literature,[15,29] is perhaps better thought of as *membrane-associated* ER, with the ERα-p85 interaction providing the first clue as to how this association might be achieved. ERα-p85 binding is consistent with the possibility that ERα is tethered with p85 to some sort of scaffold (compare Figure), such as the cytoplasmic domain of another receptor, as is common for many signal transduction pathways.[30] Furthermore, there are increasing examples of heterodimeric receptor complexes, including heterodimers between receptors of different families,[31] raising the intriguing possibility that ERs could participate similarly in heterodimeric receptor complexes.

The ER-eNOS signaling pathway has now been reported from several laboratories using many different cell types, including both human arterial and venous endothelial cells, as well as bovine and ovine endothelial cells. Cell type, and especially cell culture conditions, likely alter the level of expression of ERs in cells and the relative abundance of various isoforms. Cell culture conditions also may influence the expression of other proteins in the signaling pathways being examined. In the recent study[10] appearing in *Circulation Research* using EA.hy926 cells, the 46-kDa ER being expressed may be the hERα46 isoform of human ER recently identified by the Gannon laboratory.[32] Also, in the recent study from the Liao laboratory,[17] extraordinary levels of ERα are recovered by immunoprecipitation from nontransfected human endothelial cells. This very high level of ER protein expression has not been noted previously in any vascular cell[9,33-36] and suggests that the conditions and/or cell type used in the Liao laboratory may be uniquely suited for detecting the ERα-p85 interaction. In addition, the effects on the overall eNOS signaling system of adenoviral-mediated genetic manipulations used in various experiments need to be more completely characterized. For example, does infection with the dominant-negative Akt virus alter eNOS expression levels, or activation of eNOS by established pathways such as acetylcholine or bradykinin stimulation? These sorts of control experiments are important but are lacking in most studies published to date.

Finally, and perhaps most importantly, although in vivo evidence for the importance of the Akt pathway in vasomotion has appeared recently,[37] there is no compelling evidence at present that the nongenomic activation of eNOS by estrogen has any physiological or cardioprotective role. Under what physiological conditions would endothelial cells see the sorts of changes in estradiol similar to those required to elicit the in vitro effects observed? Is it this signaling pathway that underlies the pathophysiology of "hot flashes" during the perimenopause? It is also possible that local fluctuations in estrogen, perhaps involving pregnancy in women, or conversion of testosterone to estrogen locally by aromatase in men, may be relevant in this regard. However, the formal tests of physiological relevance for estrogen-induced vasodilation are probably best carried out next in animal models of atherosclerosis or vascular injury using pharmacological inhibitors of nitric oxide synthases and transgenic mice in which one or more NOS isoforms are fully disrupted. If the nongenomic activation of eNOS by estrogen-ER proves to be physiologically relevant, it is likely to have an important role in cardiovascular biology, because vasodilation is an important end point for a number of cardiovascular therapies for ischemic diseases, congestive heart failure, and hypertension. Nongenomic eNOS activation then would join transcriptional activation of cardiovascular target genes by estrogen as a second relevant direct effect of estrogen on vascular tissues and would provide another important end point for drug development of cardiovascular-selective estrogen receptor modulators (SERMs). Based on the rapid progress in this field of late, the visionary prediction of Samuel Levine in 1958 seems much closer to being realized.

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