Receptor-Dependent and Genomic-Independent Actions of Estrogen in Vascular Protection

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Functional evidence for a rapid, receptor-dependent and genomic-independent action of estrogen in vascular cells continues to accumulate. Although the nature of the receptor is not yet clear, some of the hormone-induced effects can be blocked by known estrogen antagonists (e.g., ICI 182,780) and can be mimicked by membrane-impermeable forms of estrogen. Because the endothelial output of nitric oxide (NO) is a major regulator of several cardiovascular functions, regulation of NO production has received a lot of attention as a potential mechanism for the cardiovascular protection offered by estrogen. There is ample evidence that estrogen can stimulate NO production and activate endothelial NO synthase (eNOS) both in *vitro* and in *vivo*. Recent investigations have shown that estrogen's rapid stimulatory action on eNOS is mediated by the activation of phosphatidylinositol 3-kinase (PI3-K) and protein kinase B (PKB)/Akt pathway among other signaling systems. Although these effects are estrogen receptor-dependent, they are rapid (on the order of a few minutes) and transcription-independent and thus represent genomic-independent but receptor-mediated effects of a steroid operating in vascular cells. In this review, recent evidence for such mechanisms is summarized, and the role of estrogen receptors *in vivo* is also briefly discussed. *(Chang Gung Med J 2002;25:636-44)*

Key words: **estrogen, nitric oxide, estrogen receptor, endothelial nitric oxide synthase, phosphatidylinositol 3-kinase, vascular functions.**

Gender differences in cardiovascular mortality and morbidity exist,[1] and estrogens confer protection against atherosclerotic disease.[2] The female hormone, estrogen (mainly 17β-estradiol), causes a significant lipid-lowering effect; however, this effect can only account for a portion (~1/3) of the atheroprotective actions of E_2.[3,4] Evidence shows that E_2 exerts direct protective effects on blood vessels including an increase in vasodilatation and a reduction in vascular injury response as well as in the development of atherosclerosis.[5] Although the general functions of estrogen (E_2) and the mechanisms through the 'classic' (genomic) estrogen receptor (ER) to bring about these functions have been extensively investigated and reviewed,[6] recent evidence indicates that a receptor-dependent but genomic-independent action of E_2 may play a role in the widely observed phenomena of vascular protection offered by E_2.[7] The goal of this brief review is thus to summarize the current understanding of this particular mode of E_2's action.

Estrogen exerts diverse effects on the cardiovascular system including systemic effects such as lowering circulating cholesterol as well as direct vascular effects such as enhancing endothelium-dependent relaxation, and the actions may occur rapidly or only after the prolonged presence of E_2.[5,8,9] Currently, two estrogen receptors (ERα and ERβ) are known,

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and both are expressed in vascular endothelial cells (ECs) and smooth muscle cells (VSMCs) and in myocardial cells.[9] They belong to the steroid/thyroid hormone superfamily of transcription factors[10] and can regulate gene expression in both E_2-dependent and -independent manners.[6] However, since the genomic action of E_2 usually requires a period of time (on the order of a few hours) to be implemented, a rapid (on the order of a few minutes) response to E_2 or a similar response to the membrane-impermeable form of E_2 (e.g., E_2 conjugated with bovine serum albumin) cannot be accounted for by the classic mode of nuclear ER's action. In this review, evidence and a potential mechanism for such a rapid, ER-dependent, yet genomic-independent action of E_2 is evaluated, with emphasis on the role of phosphatidylinositol 3-kinase (PI3-K). The physiological as well as the pathophysiological significance of ER-mediated responses are discussed.

I. Rapid, Receptor-dependent Actions of E_2

More than 20 years ago, E_2's rapid action was first demonstrated in neurons where potassium currents were activated within seconds.[11] Following these findings, several G protein-coupled receptors including μ-opioid and β-adrenergic receptors were found to be affected via rapid protein kinase C and/or indirectly by protein kinase A signaling induced by E_2 in neuroendocrine cells.[12] Recent studies of vascular ECs have presented a different picture, and several physiological effects other than the rapid electrophysiological responses have also been observed. A list of recent representative studies with cultured ECs is given in Table 1. It is clear that a major function of endothelium in the production of nitric oxide (NO) has been the focus of current investigations.

Because vascular endothelial dysfunction is recognized as a major component in most cardiovascu-

Table 1. Rapid Action of E_2 on Endothelial Cells (ECs)

System (Ref.)	Site of action	Mode of action	Results
BAEC[57]	ER (ICI sensitive)	p38β MAPK MAPKAP-2 HSP27+P	preserved stress fiber and membrane integrity
hAEC/BAEC[21]	ERα	Akt+P, eNOS+P transcription-indep. WT-sensitive	reduced leukocyte accumulation
EA. hy926EC[18]	ER (ICI-sensitive) E2BSA	Akt+P; eNOS+P LY-sensitive	NO release
PAEC[19]	ERα (ICI-sensitive)	MAPK+P transcription-indep.	NO release
HUVEC[20]	ER (ICI-sensitive)	eNOS-HSP90 association herbimycin-sensitive	NO release
HUVEC[58]	ER (ICI-sensitive)	Ca^{++} indep. transcription-indep.	basal eNOS
HUVEC[22]	surface binding ERα (ICI-sensitive) E_2BSA	ERK1/2 MAPK	NO/cGMP production
h mvEC[59]	acute response of ischemia in situ	WT-sensitive	flow cessation-induced rapid NO and Ca^{++} increase

Abbreviations: BAEC: bovine aortic EC; EA. hy926 EC: human EC cell line; hAEC: human aortic EC; PAEC: pulmonary artery EC; hmvEC: human lung microvascular EC; ICI: ER antagonist (ICI 182,780 or ICI 164,384); E2BSA: membrane impermeable ligand of E2; MAPK: mitogen-activated protein kinase; MAPK AP-2: MAPK-activated protein kinase; HSP: heat shock protein; +P: phosphorylation; WT: wortmannin and LY: LY294002: PI3-K inhibitors.

lar disorders and endothelial NO synthase (eNOS or NOS III) is vital in maintaining the integrity of endothelium, the regulation of eNOS is the key event in understanding vascular responses induced by physiological (e.g., hormones) or pathophysiological (e.g., oxidative stress) changes.[13-16] Transcriptional regulation and post-translational regulation characterized by subcellular translocation and covalent modifications such as phosphorylation, acylation, and protein-protein association with calmodulin and caveolin of the eNOS protein have been reviewed.[13,17] Table 1 illustrates that shortly (on the order of a few minutes) following E_2 stimulation both phosphorylation via mitogen-activated protein kinase (MAPK) and PI3-K protein kinase B (PKB, or Akt) signaling and protein-protein association with heat shock protein have been reported in EC. Most of these studies have also reported that these rapid actions of E_2 stimulate eNOS activity and result in a rapid release of NO.[18-22] Evidence that these actions of E_2 are ER-dependent is based on observations that they are inhibitable by ER antagonists such as ICI 182,780. Furthermore, these actions are not the consequence of classic genomic-dependent effects of nuclear ER because (1) a membrane-impermeable agonist (e.g., E2BSA) is similarly effective;[18,22] (2) a rapid response (on the order of a few minutes) is observed;[18-21,23] and (3) the actions are transcription-independent.[18,19,21] Taken together, these results strongly indicate that E_2 causes rapid, ER-dependent but genomic-independent stimulation of eNOS activity via MAPK or PI3-K/PKB signaling pathways in cultured ECs. Involvement of the activation of ionic channels, the generation of cyclic nucleotides, and GPCR signaling during the rapid E_2 action have been reviewed elsewhere.[7,24] Therefore, we only discuss recent evidence for this particular mode of signaling system, i.e., the PI3-K and PKB/Akt pathways elicited by E_2 resulting in the stimulation of eNOS and the generation of endothelium-derived NO that have often been proven responsible for the observed vascular protection.

II. PI3-K Signaling

PI3-K is a family of lipid kinases that can phosphorylate the D-3 position of the inositol ring of phosphoinositide lipid to form phosphatidylinositol (PtdIns) (3) phosphate (PtdIns (3)P), PtdIns$^{(4,5)}$-bisphosphate (PtdIns(4,5)P_2), and PtdIns(3,4,5)P_3. PI3-K can be divided into 3 classes on the basis of their *in vitro* specificity to the lipid substrate, structure, and mode of regulation: class IA and IB (all lipids as substrates), class IIC2α, (PtdIns and PtdIns(4)P as substrates), and class III (PtdIns as the only substrate). These lipids then bind to the pleckstrin homology (PH) domain of several proteins including PtdIns(3,4,5) P_3-dependent protein kinase-1 (PDK-1), serine/threonine protein kinases such as PKB/Akt, Brutons tyrosine kinase, and several guanine-nucleotide exchange proteins of the Rho family GTPases.[25-29] These molecules are activated by many extracellular stimuli and have been implicated in a wide range of cellular processes, including metabolic control, cell cycle progression, cell growth, cell motility and adhesion, and cell survival.[25,26,28,29] We concentrated on some recent studies in cultured vascular cells (ECs and VSMCs) and in isolated rat arteries to illustrate a variety of stimuli including physical challenges (e.g., balloon injury or shear stress) and hormonal factors (e.g., adrenomedullin and angiotensin II) which were found capable of activating the PI3-K signaling system and eventually leading to a protective effect often associated with enhanced NO production (Table 2). First, eNOS activity was increased through direct serine phosphorylation by PKB/Akt, indicating that the PI3-K connection in COS-7 cells (which do not express NOS) was co-transfected with eNOS and Akt as well as in mvECs,[30] bovine aortic ECs (BAECs),[31] and human umbilical vein ECs (HUVECs).[32] Second, both vascular endothelial growth factor (VEGF) and insulin were found to stimulate NO production via a PI3-K-inhibitor (wild type (WT) and/or LY)-sensitive mechanism in HUVECs,[33,34] suggesting that different stimuli utilize PI3-K signaling to activate eNOS in the same cell. Third, in VSMCs, PI3-K activation is involved in angiotensin II-induced DNA synthesis and cell proliferation through generation of reactive oxygen species (ROS), a process which is likely involved in the proliferation of VSMCs during vascular injury.[35,36] In fact, medial replication following balloon injury to rat arteries was found to be associated with PKB/Akt phosphorylation.[23] Furthermore, the finding that adrenomedullin enhances endothelium-dependent relaxation of intact rat aorta via Akt phosphorylation suggests that the PI3-K-PKB/Akt signaling system may play an important role in the homeostasis of vascular structure and function.[37]

Table 2. PI3-K Actions in Vascular Cells

System	Stimulant	Assay	Comment	Ref.
Rat aorta	adrenomedullin	Akt+P EDR	Ca^{++}/CaM dep.	52
VSMC/ rat arteries	balloon injury	PKB+P	medial replication	23
COS-7 cells mv EC	VEGF	Akt+P eNOS+P	Ca^{++}-indep. basal NO	30
VSMC	Angiotensin II	p85 (PI3-K)+P and translocation	DNA synthesis and hyperplasia	35
VSMC	Angiotensin II	Akt+P	ROS-mediated hyperplasia	36
BAEC	Sphingosine 1- phosphate	Akt+P eNOS/NO	Gβγ-regulated	60
BAEC	shear stress	Akt+P	eNOS+P	31
HUVEC	shear stress	Akt+P cGMP/EDR	eNOS+P Ca^{++}-indep.	32
HUVEC	VEGF	WT and LY-sensitive	eNOS/NO	33
HUVEC	insulin	WT-sensitive	NO production	34

Abbreviations: EC: endothelial cell; BAEC: bovine aortic EC; HUVEC: human umbilical vein EC; mv EC: bovine lung microvascular EC; Akt or PKB: protein kinase B; CaM: calmodulin; +P: phosphorylation; WT: wortmannin; LY: LY294002; VEGF: vascular endothelial growth factor; EDR: endothelium-dependent relaxation.

The evidence thus has demonstrated that the PI3-K-PKB/Akt signaling system is activated through the ER-dependent vascular action of E_2, as part of a program to stimulate eNOS for enhanced endothelial NO production in a genomic-independent manner (Table 1). In addition to the action of E_2, a host of stimuli including other hormones and shear stress also utilizes the PI3-K pathway to activate eNOS in cultured ECs as well as in arterial tissue (Table 2). The involvement of PI3-K-eNOS coupling is neither limited to E_2 action nor to ECs alone,[23,35,36] indicating the important position of PI3-K in the regulation of vascular homeostasis. Figure 1 summarizes the possibilities of ER-dependent vascular regulation mediated by E_2 which includes genomic actions, the PI3-K-eNOS pathway, and other non-genomic effects (e.g., via MAPK or Ca^{++} fluxes).[38,39] The rapid non-genomic actions of E_2 are likely to be triggered after E_2 binding to a membrane component for signal transduction. However, the nature of such a plasma membrane ER is unknown, and its molecular structures have been proposed to be a protein unrelated to nuclear ER or a nuclear ER.[40]

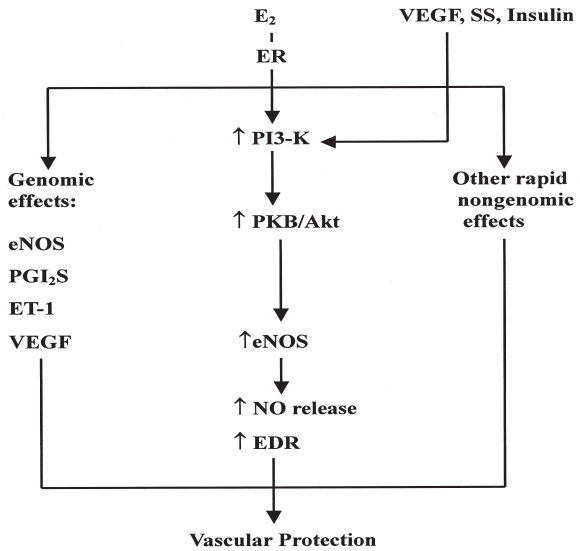

Fig. 1 Vascular protective effects of E_2. E_2 binds ER and causes both long-term genomic effects which alter several genes (e.g., eNOS and ET-1) involved in vascular regulation and rapid nongenomic effects including the PI3-K-PKB/Akt-eNOS pathway and other signaling systems. ER: estrogen receptor; SS: shear stress; VEGF: vascular endothelial growth factor; PGI2S: prostacyclin synthase; ET-1: endothelin-1; EDR: endothelium-dependent relaxation.

III. Role of Cardiovascular ER *in vivo*

The above evidence, albeit strong, is mostly derived from in vitro investigations focusing on rapid PI3-K-mediated vascular actions of E_2. With the development of transgenic animal models, the physiological and pathophysiological roles of ER have also been examined in ER knockout (KO) mice. Two estrogen receptors, ERα and ERβ, encoded in separate genes, have been characterized[41-43] and were found to be expressed in both ECs[44] and VSMCs.[45] Although early studies using an ovariectomized (OVX) ERαKO model and carotid arterial injury showed that E_2's inhibition of vascular injury was independent of ERα,[46] later investigations with transgenic animals demonstrated that ERα and/or ERβ were involved in the E_2-elicited vascular protection (Table 3). However, the results were by no means simple or unequivocal to interpret and require careful considerations of various conditions of the animal model. In a study designed for obtaining unambiguous information, ovariectomized ERα, βKO (in which both ERα and ERβ were disrupted) mice and WT littermates were investigated following carotid artery injury with or without E_2 administration.[47] It was found that E_2 inhibited the increase in the vascular medial area (as an index for injury) in WT mice but not in ERα,βKO mice, illustrating an ER-dependent mechanism of E_2 protection in reducing the extent of injury.[47] However, E_2 significantly increased the uterine weight and also inhibited proliferation of VSMC following injury to ERα,βKO mice, suggesting that either an ER-independent mechanism, an unidentified ER subtype, or residual activity of the ERα splice variant was involved in at least some of the beneficial effects of E_2.[47] These findings showed that even application to mice harboring disruptions of both ERα and ERβ genes could not resolve the multiple possibilities of ER-mediated signaling in all target tissues. Furthermore, the most recent reports from the same laboratory[47,48] have added a new player to the game. In ERβKO mice, VSMCs and blood vessels were found to exhibit

Table 3. ER-dependent Protection *in vivo*

System (Ref.)	Protective role	Comments
FKBP 12.6 nullmice (50)	cardiac hypertrophic response to Ca^{++} dysregulation	Tamoxifen sensitive; gender difference
ERβ-deficient mice (48)	endothelium-independent vasoconstriction, K^+ channel dysfunction, BP	ICI sensitive iNOS dep. both genders
Ovariectomized mice with E_2	basal NO release enhanced ACh-induced EDR reduced	ERα-dep. ERβ indep.
ERα(-/-) and ERβ(-/-)(49)	NOS protein not affected	
Ovariectomized mice also lack of apoE (51)	lesion size reduced by E_2	ERα dep. ERα(-/-)
ERα and ERβ double knock out mice (ERα,β KO) (47)	carotid artery injury reduced by E_2 in control but not in ERα,β KO	both ERα and ERβ involved
Ovariectomized rats with E_2 (61)	recovery of post-ischemic cardiac function	iNOS stimulated (Ca^{++}-indep.) cGMP increased
Ovariectomized ERα-deficient mice with E_2 (46)	carotid artery injury reduced in control and ERα KO	ERα indep.

functional abnormalities, and E_2 augmented vasoconstriction instead of vasorelaxation, which may be related to iNOS and the potassium ion channel.[48] Both systolic and diastolic blood pressure increased in ERβKO mice, indicating that ERβ may control important functions of vascular physiology involving iNOS in both genders.[49] Interestingly, in primary human VSMCs, transfection with ERβ or ERα resulted in the opposite effect on the iNOS reporter gene, and transfection of both ERs resulted in intermediate activation of the reporter.[48] It has also been shown that basal NO production was increased in OVX-ERβKO mice in response to E_2, whereas this effect was abolished in OVX-ERβKO mice,[49] suggesting that it is ERα, not ERβ, that mediates the beneficial effect of E_2 on basal NO production. Therefore, it appears that E_2's protective effect on vascular functions may involve both ERα and ERβ as well as both eNOS and iNOS; it may exert its effects on both endothelium-dependent and -independent parameters,[48,49] and at times, the actions of ERα and ERβ (e.g., iNOS induction) may antagonize each other.[48] Whether the rapid PI3-K-eNOS pathway is also involved in the long-term ER-dependent action of E_2 has not been directly examined yet. Table 3 also lists a couple of examples illustrating E_2's cardioprotective actions.[50,51]

IV. Conclusions

Vascular endothelial and smooth muscle cells exhibit ER in both women and men,[9] and ERα activates specific target genes (Fig. 1). In addition, rapid nongenomic effects involving ERα (Tables 1, 2) do not require alterations in gene expression but may activate a rapid signaling system, e.g., PI3-K-PKB/Akt[18,21] or a protein-protein association, e.g., ER and HSP90,[20] to induce vasodilatation via the eNOS-NO pathway. In postmenopausal women, E_2 causes short-term dilatation of coronary and brachial arteries, mediated largely by the enhanced production of NO. This and other findings[8,9] indicate that rapid ER-dependent activation of eNOS is consistent with the observed vasodilatation and may play an important role in the benefits associated with E_2.

The observed gender difference in vascular physiology and the protective role of E_2 in pathophysiology are interesting and elaborate, with many confounding factors. The multiplicity of interrelated elements, including social, personal, systemic, and local (vascular cell) origins,[52] renders experimental approaches to understanding E_2's protective actions difficult, sometimes even elusive. Although some 40 observational studies have suggested that women who take E_2 or hormone replacement therapy (HRT) exhibit a lower risk of coronary heart disease (CHD) than non-takers,[53,54] randomized trials with preexisting CHD and secondary prevention trials have not confirmed the cardioprotective effect of E_2 or HRT.[55] The recent AHA Scientific Statement[56] should be consulted for readers who are interested in these apparent discrepancies.

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雌激素引發受體依賴性但非基因體依賴性之血管保護作用

樓迎統

雌激素可以經由受體媒介，但係非基因體依賴性的快速作用引起很多興趣。雖然雌激素受體本質仍不清楚，快速作用可以被受體拮抗劑抑制，也可以被不能通過細胞膜之雌激素型劑模倣。因爲內皮膜產生一氧化氮 (NO) 是重要調控心血管的機制，雌激素保護心血管的機轉中也以增加NO生產爲主要管道。在活體或體外實驗中，不少證據顯示雌激素活化內皮NO合成酶 (eNOS) 刺激NO產生。近來研究更發現雌激素經快速活化磷脂激酶 (PI3-K) 訊息傳遞系統，刺激內皮NO合成。這些觀察代表在血管細胞中雌激素快速且經受體媒介 (但非轉錄依賴性) 的作用可能參與保護心血管。本文回顧最近的相關證據並討論血管雌激素受體之生理意義。(長庚醫誌 2002;25:636-44)

關鍵字：雌激素，一氧化氮，雌激素受體，內皮一氧化氮合成，磷脂激酶，血管功能。

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