Endothelial Dependent Dilation by Estrogen through the AKT / PKB Pathway.

Maria Florian

Department of Physiology, McGill University,
Montreal (Québec), Canada

October 2001

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Masters of Sciences.

TABLE OF CONTENTS

Figures Legends and Figures

Schema Legend

Figure Legends

Appendices

Schema

List of Figures

Summary

Acute administration of estrogen results in the vasodilatation and in the release of nitric oxide (NO) that occurs through activation of the serine-threonine kinase Akt/protein-kinase-B (PKB), which is known to increase the eNOS activity. 10^{-8} M of 17-ß-estradiol resulted in a left shift of the vasodilatory response to Ach in preconstricted aortic rings from oophorectomized rats ($EC_{50} = 0.7 \times 10^{-8}$ M with 17-ß-estradiol and 0.15×10^{-7} M of Ach without 17-ß-estradiol, $P<0.05$). The effect was blocked by pre-treatment with Wortmannin, a PI(3)K inhibitor. AKT / PKB was phosphorylated in endothelial cells (EC) as early as 1-minute after estradiol-stimulation. Phosphorylation of eNOS and NO release in EC treated with 17-ß-estradiol were also increased. We conclude that the AKT/PKB pathway is involved in the acute release of NO by estrogen.

Résumé

L'administration aiguë d'œstrogènes entraîne la vasodilatation et la libération de monoxyde d'azote (NO). Cette dernière est causée par l'activation de la voie de la serine-threonine kinase Akt/protéine-kinase-B, qui serait impliquée dans l'augmentation de l'activité de la eNOS. 10^{-8} M de 17-β-œstradiol a causé le déplacement vers la gauche de la courbe dose-réponse de vasodilatation à l'Ach des segments d'aorte précontractés, de rats ovariectomisés (EC_{50}=0,7x10^{-8} M et 0,15x10^{-7} M avec et sans 17-β-œstradiol respectivement, $p<0,05$). Cet effet est aboli par un prétraitement avec le Wortmannin, un inhibiteur de la PI(3)K. L'AKT/PKB est activée dans les cellules endothéliales (CE) dès la première minute de stimulation à l'œstradiol. La phosphorylation de la eNOS et le dégagement de NO dans les CE traitées avec 17-β-œstradiol, ont aussi augmenté. La voie de la AKT/PKB participe à l'action des œstrogènes sur la biodisponibilité du NO.

Preface

Since starting my Masters in Physiology, within the Critical Care Division of the Royal Victoria Hospital, I have contributed to the following publications and presentations:

Publications

Fencheng Ye, **M. Florian**, S.Magder, S.Hussain
Regulation of Angiopoietin and Tie-2 Receptor Expression in Non-reproductive Tissues by Estrogen.
Steroids. Vol 66. 2001, [manuscript in press].

M. Florian, Rosario Leonor, S. Magder
Endothelial Dependent Dilation by Estrogen Through the AKT/PKB Pathway.
Supplement to Circulation. Vol 102, No 18, p. II-106. October 31, 2000.

M. Florian, S. Magder
Estrogen Decreases Superoxide Production in Rat Aortic Tissue.
Circulation. Supplement I. Vol 100, No 18, p. I-266. November 2, 1999.

M. Florian, S. Magder
Estrogen Decreases Superoxide Production in Rat Aortic Tissue.
Can J Cardiol. Supplement D. September 1999. Vol 15: 147 D.

A.Freiman, **M. Florian**, D. Javeshghani, S. Magder
Estrogen Therapy Does Not Alter ec-NOS Protein in Oophorectomized Rats.
Can J Cardiol 14:96F. September 1998.

Presentations

M. Florian, S. Magder
A Decrease in Superoxide Production with Estrogens in Oophorectomized Rats.
6th Mini-Symposium F.R.S.Q. Sub-Network: Endothelium And Its Vascular-Related Diseases. Montreal. Poster session. March 4-5, 2001. 1st place for best poster presentation.

M. Florian, Rosario Leonor, S. Magder
Endothelial Dependent Dilation by Estrogen Through the AKT/PKB Pathway.
American Heart Association, 73[d] Scientific Sessions, New Orleans, Oral Abstract Presentation. November 2000.

S. Magder, **M. Florian**
Genomic and Non-genomic Actions of Estrogen on the Vascular Wall.
Cardiovascular axis mini-retreat, Montreal, Oral Presentation. September 2000.

M. Florian, S. Magder
Estrogen Decreases Superoxide Production in Rat Aortic Tissue.
American Heart Association, 72[nd] Scientific Sessions, Atlanta. Poster session. November 1999.

M. Florian, S. Magder
Estrogen Decreases Superoxide Production in Rat Aortic Tissue.
Canadian Cardiovascular Society, 52[nd] Annual Meeting, Quebec City. Poster session. October 1999.

A. Freiman, **M. Florian**, D. Javeshghani, S. Magder
Estrogen Therapy does not alter ec-NOS protein in oophorectomized rats.
Canadian Cardiovascular Society, 51[st] Annual Meeting, Ottawa. Poster session. October 1998.

Rosario Sabrina Leonor, an undergraduate student from the Department of Physiology (McGill University), was responsible for the experiment of the rat aorta contractility.

This study was selected for an oral abstract presentation at the 73[d] Scientific Sessions of the American Heart Association in November 2000 and was published as a supplement to the October 2000 issue of Circulation (*see Appendices*). The manuscript is currently being submitted for a publication.

Acknowledgments

First and foremost, I would like to express my gratitude to my supervisor, Dr Sheldon Magder, for providing me with the opportunity to undertake this project in the Critical Care Division of the Royal Victoria Hospital. I am grateful to him for his patience in guiding me throughout my research training, for financial support and for the great opportunities to participate in seminars and congresses. It has been my privilege to work in his laboratory where I found many sources of motivation.

I wish to extend a special thanks to Dr Sabbah Hussain for his most valuable recommendations and insight through my research work as well as for his tolerance and benevolence.

I will always appreciate the support and encouragement of Linda Tracey, Christine Pamplin and members of the Physiology Department at McGill University.

In particular, I would like to thank Jeanna Neculcea for introducing me to the laboratory work and especially for teaching me cell culture techniques. I am grateful for her assistance with cultured cell assays.

I would like to extend special thanks to Dr Alain Comtois for his intellectual advice and generous help.

I thank Luigi Franchi, who was always ready to discuss the strangest western immunoblotting as well as to help me with many other daily lab tribulations.

I am also endlessly grateful to Christine Mutter, Roberta Carin, Joan Longo and Jennifer of the administration office in Critical Care Division for their precious and efficient help with everything.

I would like to thank the many people whose contribution I greatly appreciated: Doris Grienke for animal care and training, Anne-Claire Lukasziewicz for the French translation of my abstract, Danesh Javeshghani, Yan Guo and Fengchun Ye for contributing with their mature opinions on experiments; I also acknowledge the pleasant company of my fellows students, Anatolij, Jonathan, Khaled, Esther, Rania, Moheb, Theo, Dimitri and Imad.

I feel fortunate to have the love and support of my endlessly patient family. In particular, I am grateful to my husband, who understood and approved the necessity to reorient my career, and to my dearest and faithful daughter, who was always enthusiastic about my work; lastly, to my far away family for the encouragements and for its trust in me.

Abbreviations

Ach: acetylcholine

AKT/PKB: serine/threonine kinase-AKT/protein kinase-B

Ang II: angiotensin II

BAEC: bovine aortic endothelial cells

BAMEC: bovine adrenal medulla endothelial cells

BH_4: tetrahydrobiopterin

BMEC: bovine microvascular endothelial cell

cAMP: cyclic adenylate monophosphate

cGMP: cyclic guanylate monophosphate

CHO: Chinese hamster ovary

COS-7: continuous cell line, hosts for transfection

E: 17-β-estradiol

E_2BSA: membrane-impermeant β-estradiol-17-hemisuccinate conjugated to bovine serum albumin

EA.hy926: HUVEC-sarcoma fusion cell line

EC: endothelial cells

eNOS : endothelial nitric oxide synthase

ER-α: estrogen receptor α

ER-β: estrogen receptor β

ER(s): estrogen receptor(s)

ERE: estrogen response element

Erk: extracellular signal regulated kinase

ERKO: estrogen receptor knockout mice

GC: guanylate cyclase

HBD: hormone-binding domain

HMG-CoA: 3-hydroxyl-3-methyl coenzyme A reductase

Hsp27: heat shock protein 27

Hsp90: heat shock protein 90

HUVEC: human umbilical vein endothelial cells

IGF-1: insulin like growth factor-1

iNOS: inducible nitric oxide synthase

LBD: ligand-binding domain

L-NAME: N^{ω}-nitro-L-arginine-methyl-ester

L-NMMA : L-N^{G}-monomethyl-L-arginine

MAPK: mitogen activated protein kinase

MAPKAP-2 kinase: mitogen-activated protein kinase-activated protein

MCF-7: human mammary cancer cells

NAD(P)H: nicotineamide adenine dinucleotide (phosphate)

NMDA *:* *N*-methyl-D-aspartate

nNOS : neuronal nitric oxide synthase

NO: nitric oxide

NOS: nitric oxide synthase

O_2^-: superoxide

$ONOO^-$: peroxinitrite

PAEC: pulmonary artery endothelial cells

PDK1, PDK2: protein dependent kinase-1 (2)

PH: pleckstrin homology

Phe: phenylephrine

PI(3)K: phosphatidylinositol-3-OH-kinase

PIP2: phosphatidylinositol-bisphosphate

PIP3: phosphatidylinositol-trisphosphate

PKB: protein kinase-B

PtdIns(3,4)P_2: phosphatidylinositol-3,4-bisphosphate

PtdIns(3,4,5)P_3: phosphatidylinositol-3,4,5-triphosphate

PtdIns: phosphoinositides

PtdIns3: 3'-phosphorylated phosphatidylinositides

PtdIns3*P*: 3'-phosphorylated phospholipids

SDS-PAGE : sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Ser^{473}: serine 473

SMCs: smooth muscle cells

Shc: adapter protein

Src: tyrosine kinase

SOD: superoxide dismutase

Thr^{308}: threonine 308

TMX: tamoxifen

TRLEC: simian virus 40-transformed rat lung vascular endothelial cells

VEGF: vascular endothelial growth factor

VSMC : vascular smooth muscle cells

W: wortmannine

WT : wild type

1. Introduction and Literature Review

The incidence of cardiovascular disease differs significantly between men and women, but the mechanism is not known. The incidence of atherosclerotic diseases is low in premenopausal women and is increased in postmenopausal women but is nonetheless markedly reduced in postmenopausal women receiving estrogen therapy (1). However, some recent large observational studies, the Heart and Estrogen / Progestin Replacement Study (HERS) (2), Estrogen Replacement and Arherosclerosis randomized trial (ERA) (3) and some other studies (4-6), have not confirmed the previously proposed protective effect of estrogens .

The atheroprotective effects of estrogen were initially attributed principally to lipid-lowering effects of estrogens (1). This, however, only explains part of the benefits (7). Recent data suggest that direct actions of estrogen on blood vessels contribute substantially to their cardiovascular protective effects (8). Estrogen affects the vessel wall by two different general mechanisms: it has rapid, non-genomic vasodilatory effects, and slower long-term effects, which are through the traditional genomic actions of estrogen. An important part of the effect of estrogen actions on the vasculature is through an increase in endothelium-derived bioavailable NO, which may be both through genomic and non-genomic mechanisms (7).

1.1. Genomic effect of estrogen (Transcriptional regulation)

Genomic effects of estrogens require hours to days for a modification in gene expression to occur. The classical mode of action of a steroid hormone, such as estrogen, is by the interaction of the activated receptor with the promoter of different genes via activated steroid receptors and by transcriptional upregulation. The ligand-activated estrogen receptor (ER) acts as a transcriptional factor (9). The attachment of estrogen to the receptor causes activation and dimerization of receptors. The palindromic DNA target sequence, within estrogen-responsive genes, is called an estrogen response element (ERE). For example, the gene for nitric oxide synthase

(NOS) contains 11 copies of the half-palindrome EREs often associated with an Sp1 site (10;11). Estrogen increases the expression of genes for important vasodilator enzymes such as NOS. Some of the rapid effects of estrogen may therefore be due to longer-term increases in the expression of the genes for NOS in vascular tissues. Long-term administration of estrogen increases Ach-mediated coronary vasodilatation. The preventive effect of estrogen against atherosclerosis occurs over a period of hours to days after estrogen treatment and is likely therefore at least partly dependent on changes in gene expression in the vascular tissue.

1.2. Non-genomic effects of estrogen (Posttranscriptional regulation)

Besides transcriptional activities of estrogens, there is also increasing evidence for its transcription-independent actions in target cells (12). The rapid effect of estrogens was shown in osteoblasts and osteocytes (12;13), breast cancer cells (14) and cerebral cortical cells (15;16).

The non-transcriptional effects of estrogen are mediated by membrane bound ERs (17-20). The nongenomic stimulation by estrogen takes place through modulation in the activity of signal transduction cascades, such as Src-Shc-Erk pathway (13;14;16;20). 17-β-estradiol, stimulates tyrosine kinase and within seconds induces protein phosphorylation (14;21;22).

Similarly to the effects of the growth factors, such as IGF-1, estrogen stimulates phosphorylation of the antiapoptotic serine/threonine AKT/PKB pathway (23). Rapid stimulations of EC by VEGF (24), by shear stress (25) and by estrogen (19;26;27), activate PI(3)K and AKT kinase, induce downstream eNOS activity (17-19;26-28) and subsequently leads to release of NO (24;25).

Estrogens mediate non-transcriptional effects in cells within seconds (14). Estrogen-induced vasodilatation occurs 5 to 20 minutes after administration. This is too fast to be dependent on changes in gene expression and therefore must involve a *non-genomic* mechanism (8). For example, acute administration of estradiol produces a rapid endothelium-dependent dilation of normal vessels and atherosclerotic arteries which is believed to be dependent on the release of NO (8;29;30). As well, acute

estrogen exposure causes, within 20 minutes, and endothelium-dependent coronary dilation in female primates (31). Estrogen administration also prevents coronary arterial constriction in response to Ach in post-menopausal women (32).

In postmenopausal women with coronary artery disease, acute administration of 17-β-estradiol caused within 20 minutes of exposure, a vasodilatation of the coronary artery. This effect was not apparent in men of a similar age with the same pathological condition (32). Sublingual administration of 1 mg of 17-β-estradiol to postmenopausal women, produced an acute delay of exercise-induced myocardial ischaemia, which gives indirect evidence for a vasodilatory effect of estrogen in postmenopaual women (33). The mechanism, by which estrogen appears to inhibit the development of atherosclerosis in females, is through the increased formation of NO in vascular endothelium.

A direct vasodilating effect of estrogen at pharmacological doses, was observed on the rat tail artery (34).

Estrogen also causes a rapid release of NO from cultured EC. This action is mediated by plasma-membrane located estrogen receptors, inhibitable by specific ER antagonist (18). In bovine aortic endothelial cells (BAEC) treated for 30 seconds with 17-β-estradiol, there is a 3-fold increase in NO generation resulting from functional activation of endothelial nitric oxide synthase (eNOS) (35). The short-term effects of estradiol-mediated NO release, however, did not result from an increase in eNOS protein expression (28).

In summary, vascular effects of estrogen are mediated at least in part through NO (36) and are associated with increased production of NO (37).

Because NO has a multiple range of functions in biological systems, I will give here a short outline of the nature of NO.

2. Biology of Nitric Oxide

2.1. Main characterisctics of NO.

NO is a small lipophilic and chemically unstable molecule, which easily diffuses out of one cell into one or more adjacent cells. A large source of NO synthesis is from the vascular lining. NO is synthesized not only by vascular EC but also can be synthesized by macrophages, neutrophils, hepatic Kupffer cells and brain tissue.
NO also plays a physiological role in local transcellular communication. For example, NO acts as a potent activator of cytosolic guanylate cyclase (GC) and elevates tissue cyclic guanylate monophosphate (cGMP) levels (38). Under these conditions NO has been shown to stimulate c-GMP-dependent protein kinases which leads to increased activity of Ca^{2+}-dependent K^+ (K_{Ca}) channels (39-43). Several chemical messengers, such as hormones and Ach, can activate NOS in cells, by binding to appropriate receptors on the cell membrane. In vascular beds the NO formed by the EC, diffuses freely into both the interior of the blood vessel and into underlying smooth muscle cells (SMCs), where the NO can exert its effects (44).

2.2. Synthesis of nitric oxide.

The free radical NO is generated exclusively by the enzyme NOS. NO is synthesized from the amino acid L-arginine through a five-electron oxidation step via the formation of the intermediate N^G-hydroxy-L-arginine. The conversion of L-arginine to NO is specific because a number of analogues of L-arginine are not substrates, including its D-enantiomer. NOS-mediated NO production requires the presence of NADPH, tetrahydrobiopterin (BH_4) and free Ca^{2+}. The enzyme NOS contains binding sites for heme and calmodulin, which are essential for the production of citrulline and NO. NO binds to iron in the heme and activates GC. An increase in intracellular calcium stimulates the constitutive NOS to form NO from L-arginine (45). As well,

NO synthesis can be abolished by L- N^G-monomethyl-L-arginine (L-NMMA), which inhibits both the production of citrulline and the increase in cyclic GMP (46).

2.3. Biological role of NO in a vasculature

NO is a major endogenous vasodilator, which is produced by vascular endothelium. Many investigators have showed the effect of NO release on vascular tone (10). Initially, NO was named endothelium-derived relaxing factor (38). NO also contributes to the control of platelet aggregation, the regulation of cardiac contractility and adhesion molecules. Intact endothelium releases NO in response to numerous agonists such as Ach and causes vasodilatation (47). Isolated preparations of intact arteries, precontracted with norepinephrine, exhibit relaxation to the agonist Ach. Ach acts on muscarinic receptor on EC. This increases intracellular calcium, which activates NOS. In the rat aorta, endothelium-dependent vasorelaxation produced by Ach is abolished by NOS inhibitors, indicating that the vasodilatation is mediated by NO.

NO mediated vasodilatation is diminished in the forearm arteries of patients with essential hypertension, and it decreases with increasing blood pressure. As well, systemic vasodilatation evoked by hypoxia is prevented by L-NMMA (48). Thus, NO is a major factor regulating peripheral resistance.

One of the first observations of NO mediated vasorelaxation (49), was increased basal release of NO during vasodilatation in response to SOD, which is an O_2^- scavenger, that decreases the breakdown of NO (50). Scavenging of NO by O_2^- results in an attenuation of NO signaling mechanism, such as those mediated through cGMP. In tissues, the interaction of NO with O_2^- results in the production of peroxynitrite ($ONOO^-$) (51).

2.4. NOS isoforms

There are three, highly homologous isoforms of NOS. Two isoforms are constitutively expressed, although their expression may be modulated: nNOS (or NOS1) is expressed primarily in neurons, and eNOS (NOS3) is expressed in EC, cardiac myocytes and blood platelets. The expression of iNOS (NOS2) is induced by various cytokines (52). Constitutive isoforms of NOS are activated by Ca^{2+}/ calmodulin. In neurons, glycine and glutamate of *N*-methyl-D-aspartate (NMDA) receptors, allows Ca^{2+} to enter the cells. In EC, a variety of vasodilatory compounds including bradykinin, adenosine, Ach, leukotriene D_4, histamine, dopamine and the calcium ionophore A23187, increase NO synthesis. NO synthesis depends on the availability of L-arginine, BH_4 and NADPH (53).

3. Estrogen Receptors

There are two estrogen receptors, estrogen receptor α (ER-α) and estrogen receptor β (ER-β), both of which are members of the superfamily of steroid hormone receptors. Estrogen receptors α and β have considerable homology in the DNA-binding domain (96%) and in the hormone-binding domain (HBD)(53%) including hormone-dependent transcriptional-activation function. The amino-terminal domains of the ERs which contain a ligand-independent transcriptional-activation function, is unique for each of the ERs. ERs are transcription factors that alter gene expression when they are activated. ER-α is expressed mainly in vascular smooth muscle and endothelial cells, ER-β is found in many other tissues (prostate, uterus, lung, brain). Functional ER-β is found in myocardial cells, where it regulates the expression of NOS. Estrogen-estrogen receptor complexes form homodimers and bind to specific gene sites. In addition, ERs α and β can form heterodimers with each other (54).

3.1. Background

Earlier studies described the presence of an ER in human coronary arteries and human umbilical vein endothelial cells (HUVEC). The ER antagonist ICI 182,780 reduces estrogen enhancement of human EC proliferation (55). This effect was shown by [^{3}H]-thymidine incorporation assay. Estradiol-treated cells had twice the incorporation of hormone-free cells. The increase in [^{3}H]-thymidine incorporation was prevented by adding the ER antagonist ICI 182,780 to the culture medium along with the estradiol (55). The potential of EC to constitutively possess the potential for transcriptional regulation of target genes by estrogen was demonstrated on HUVEC. Cell lysates were separated into nuclear and cytosolic fractions and were subjected to standard SDS-PAGE. A protein fraction with a molecular mass of 67 kDA was identified by anti-ER antibody in the nuclear extracts from bovine and human EC. The cytosolic fractions from all cell types, however, lack any distinctive

immunostaining band for ERs. All EC types studied to date have demonstrated the presence of abundant mRNA for the ER (56).

A novel nuclear receptor, the clone 29 was isolated from a rat prostate cDNA library. Clone 29 protein is highly homologous to the rat ER protein in the DNA-binding domain (95%) and in the C-terminal ligand-binding domain (55%). Clone 29cDNA encodes a novel rat ER, which iss present in the secretory epithelial cells of the rat prostate and also in granulose cells of the rat ovary. It was subsequently named ER-β to distinguish it from the previously cloned ER (ER-α) from rat uterus (57). Tissue distribution of ERs in the wild type (WT) and ER-α knockout (ERKO) mouse was established by RNAse protection assay. WT uterus showed the greatest concentration of ER-α, whereas WT ovary possessed the highest level of ER-β. Adult females did not express ER-β in mammary glands and aorta but had slightly detectable levels of ER-βmRNA in the hearts of both genders. (58) Endothelial denudation of rat carotid artery resulted in a substantial upregulation of ER-βmRNA whereas ER-α expression in the vascular intima increased only slightly. Because of preferential ligand binding affinities of ER-β to the vascular tissue, this suggests it may play a vasculoprotective role (59).

3.2. Estrogen receptors in non-genomic actions

The role of ER-α in the rapid response to 17-β-estradiol was determined by overexpressing ER-α in pulmonary artery endothelial cells (PAEC) with ER-α cDNA. In the presence of 17-β-estradiol, there was almost a 5-fold augmentation of NOS activity as measured by [^{3}H]-L-arginine conversion assay after ER-α transfection. 17-β-estradiol caused a rapid increase in mitogen activated protein kinase (MAPK) activity in PAEC, and this effect was prevented by two ER-antagonists. A similar approach was used in COS-7 cells which do not constitutively express either ER or eNOS and thus are not estrogen responsive. The transfection of COS-7 cells with both eNOS and ER-α results in more than threefold increase in eNOS activity upon acute stimulation with 17-β-estradiol. This effect was completely

inhibited by a concomitant treatment with ICI 182,780. These data indicate that the acute stimulation of eNOS by 17-β-estradiol and ER-α entails the activation of tyrosine kinase/MAP kinase. The present observations indicate that ER-α may mediate responses in a non-genomic fashion, independently of its known ability to control transcription (18).

The effect of ER-α overexpression on basal eNOS activity transfected with sham plasmid or ER-α cDNA was measured by [^{3}H]-L-arginine conversion to [^{3}H]-L-citrulline assay in intact nonstimulated cells. Basal eNOS activity was unchanged by ER-α overexpression. In addition, immunoblot analysis revealed that eNOS protein abundance was not affected by ER-α overexpression.

Experiments were performed to determine the effect of enhanced ER-α expression on the acute response to 17-β-estradiol. The acute response (measured as % of basal NOS activity) to 17-β-estradiol stimulation was augmented four to fivefold in cells transfected with ER-α compared to sham-transfected cells. The enhanced response was inhibited completely by a concomitant acute treatment with 10 μM of ICI 182,780. These findings indicate that ER-α is capable of mediating the acute response. The inhibitory effects of ICI 182,780 and Tamoxifen (TMX), which both cause ER antagonism via interaction with the ER HBD, support the conclusion that HBD of ER-α is required for the acute activation of eNOS by 17-β-estradiol. As well, in COS-7 cells transfected with both ER-α and eNOS there was a more than threefold increase in eNOS activity upon acute stimulation with 17-β-estradiol, whereas there was no response in cells transfected with eNOS alone. As in native EC, the rapid activation of eNOS by 17-β-estradiol in COS-7 cotransfected with either ER-α and eNOS was abrogated by specific ER inhibitors indicating that the response is mediated by acute ER-α activation. These findings confirm the role of ER-α in a rapid non genomic activation of eNOS in cell types that do not constitutively express either ER or eNOS and are not estrogen responsive.

Further, the role of extracellular calcium was tested. The inhibition of calcium influx in H441 cells did not affect basal NOS activity, but fully blocked 17-β-estradiol stimulated NOS activity. This is in controversy with reports of others (19;24;25)

indicating that acute activation of NOS in EC represents Ca^{2+} independent regulatory mechanism for activation of eNOS. Studies in aortic endothelium have revealed that estrogen acutely activates calcium-dependent potassium channels, resulting in a slow increase in intracellular calcium levels through leaky membrane calcium channels (41). The potential role of tyrosine-kinase-MAP kinase signaling in acute ER-α mediated eNOS activation, has also been evaluated (60).

Engagement of cell membrane ERs in rapid endothelial NO release through a phosphatidylinositol-3-OH--kinase-AKT-dependent (PI3K/AKT) pathway has been demonstrated. A membrane-impermeant 17-β-estradiol conjugated to bovine serum albumin (β-estradiol-17-hemisuccinate) (E_2BSA) has been used to detect surface binding sites for 17-β-estradiol Cell monolayers (EA.hy926 endothelial cell line) were stimulated with E_2BSA or vehicle. Cell lysates were subjected to SDS-PAGE, probed with antiphospho-AKT antibody and reprobed with anti-AKT antibody. E_2BSA triggered AKT phosphorylation on serine 1177. A recruitment of cell-surface ER corresponded with consequent increase in NO release. Cells, overexpressing kinase-inactive AKT, did not generate E_2BSA-stimulated NO production. PI3-kinase-dependent AKT activation was not Ca^{2+}-dependent. Specific Ser1177 phosphorylation enhances eNOS Ca^{2+} sensitivity. Consequently, estrogen stimulated endothelial NO generation does not require a rise in free intracellular Ca^{2+} (19).

ER-α and eNOS are organized and functionally linked in plasmalemmal caveolae. Acute (15–60 minutes) stimulation with 17-β-estradiol caused ER-dependent activation of eNOS in plasma membranes isolated from PAEC. The response in plasma membranes was elicited without the addition of exogenous calcium, calmodulin or eNOS cofactors during membrane preparation or the incubation for NOS activity suggesting that increase in eNOS activity does not require a global rise in cytosolic free calcium (17).

In cultured bovine aortic endothelial cells (BAEC), plasmalemmal caveolae-associated ER-α mediated the acute 17-β-estradiol stimulated NO production, which was Ca^{2+}-dependent. Following E_2BSA treatment of BAEC, the existence of putative binding components in the plasma membrane was observed by fluorescence

microscopy. Pretreatment of cells with the weak ER-α antagonist TMX (1 μM) did not inhibit 17-β-estradiol stimulated NO production, while the pure ER-α antagonist ICI 182,780 (10 μM) completely attenuated 17-β-estradiol induced endothelial NO formation (28).

Because the isolation and structural characterization of the membrane ER were not defined, Chinese hamster ovary (CHO) cells, which normally do not express ER, were transfected with cDNAs for ER-α and ER-β. By Northern analysis, transcripts for ER-α or ER-β were detected. Cross-linking studies with radiolabeled 17-β-estradiol bound to CHO-ER-α or CHO-ER-β were performed to identify both membrane and nuclear receptors in pellets separated by SDS-PAGE. Proteins corresponding to 69 kDa for ER-α and 60 kDa for ER-β were found in both, membrane and nuclear fractions. This suggests, that both cell membrane and nuclear receptor proteins result from expression of single cDNA for either ER subtype.

Further, to determine whether membrane ERs are G-protein-coupled receptors, cyclic adenylate monophosphate (cAMP) was measured in membranes prepared from either CHO-ER-α or CHO-ER-β. There was significant increase in cAMP activity in preparations of both receptors when they were stimulated with 17-β-estradiol in physiological concentration compared to inactive controls (20). Interestingly, both specific ER inhibitors, ICI and TMX, did not significantly inhibit cAMP activity and actually significantly stimulated the adenylate cyclase activity. These ambiguous findings indicate that ICI 182,780 is capable of inhibiting ER-mediated G-protein activation but can also stimulate adenylate cyclase, perhaps by reducing the Michaelis-Menton constant (Km) of the enzyme. Alternatively, ICI might prevent cAMP degradation. G-protein activation by 17-β-estradiol stimulation in CHO-ER-α or CHO-ER-β was assessed by binding of the non-hydrolyzable G-protein agonist guanosine 5'-3-O-(thio) triphosphate (GTPγS) toGαs or Gαq. In both ER-expressing CHO, Gs and Gq protein activation was increased, and this was inhibited by the ER antagonist ICI 182,780. Incubation with 17-α-estradiol had no effect on GTPγS binding to the G-proteins in CHO-ER-α or CHO-ER-β membranes. It has also been

shown that a single cDNA transcript results in the production of both membrane and nuclear receptors and that membrane ERs are linked to G-protein (20).

ER-α interacted with p85α, a PI(3)K subunit, in a ligand-dependent manner in non-transfected EC and $p85\alpha^{-/-}$ fibroblasts transfected with ER-α and p85α cDNAs. This interaction is blocked by ER specific inhibitor ICI 182,780 and is absent in $p85\alpha^{-/-}$ fibroblasts transfected with ER-α cDNA alone. This effect was not confirmed in ER-β which did not interact with p85α and did not recruit PI(3)K activity after estradiol stimulation. Links between PI(3)K and ER suggest that ER may be involved in reactions outside the nucleus (27). The effects of E_2BSA, a membrane impermeable conjugate of 17-β-estradiol, and actinomycin D, an inhibitor of gene transcription, were tested in HUVEC and simian virus 40-transformed rat lung vascular endothelial cells (TRLEC) to rule out the influence of nuclear ERs. NOS activity increased similarly by treatment with E_2BSA, as with 17-β-estradiol and was not altered by actinomycin D. This indicates that the rapid 17-β-estradiol activation of eNOS does not require the classical nuclear effects of the estrogens. CHO cells were transiently transfected with cDNAs for estrogen receptors ER-α and ER-β. 17-β-estradiol stimulated the activation of AKT in CHO cells expressing ER-α but not in CHO cells expressing ER-β. These findings suggest that ER-α is capable of mediating the acute response and rapid vasodilatation caused by estrogen (26).

The role of membrane ER in the vascular protective actions of estrogen was shown on the rapid activation of heat shock protein 27 (Hsp27) by 17-β-estradiol via mitogen-activated protein kinase-activated protein serine/threonine kinase-2 (MAPKAP-2 kinase) in primary cultures of BAEC (19). E_2BSA within 10 minutes induced activation of the p38β member of the MAP-kinases and lead to the activation of MAPKAP-2 kinase and the phosphorylation of Hsp27. Further support for the role of membrane bound ER in the modulation of signaling, is that there are no differences between the effects of E_2BSA and 17-β-estradiol by *in vitro* assay of kinase activities. ER specific antagonist reversed the effects of 17-β-estradiol. E_2BSA and 17-β-estradiol stimulated the phosphorylation of endogeneous HSP27 via p38β-kinase

BAEC transfected with dominant-negative adenoviral vector MAPKAP-2 kinase, or a phosphorylation mutant HSP27 (61).

3.2.1. Tyrosine phosphorylation

Protein tyrosine phosphorylation is an important step in cellular signal transduction of estrogen. This process is actively regulated by protein tyrosine kinase and phosphotyrosine kinase phosphatase. Tyrosine also phosphorylates estrogen receptors (21).

Indeed, rapid stimulation of tyrosine phosphorylation is observed in cells treated with 17-β-estradiol for only 10 seconds (22) to 2 minutes (14). Stimulation of protein tyrosine phosphorylation of human mammary cancer (MCF-7) cells peaked at 10 seconds and declined toward the basal level 60 minutes after treatment. This action of 17-β-estradiol requires its occupancy of the receptor. 17-β-estradiol does not affect tyrosine phosphorylation of nontarget cells. Furthermore, other steroids do not share this effect on tyrosine phosphorylation (22). 17-β-estradiol in MCF-7 cells immediately and reversibly affects tyrosine phosphorylation of MAP-kinases, erk-1 and erk-2, and its upstream associated protein $p21^{ras}$. Erk-2 kinase activity is very low or absent in COS-7 cells, which do not express ER, and the level of this activity is unaffected by 17-β-estradiol treatment. In contrast, when the COS-7 cells were transiently transfected with human estrogen receptor-encoding plasmid, the kinase activity was increased upon 17-β-estradiol treatment (14).

Tyrosine kinase inhibitors attenuate 17-β-estradiol-induced rapid increase in eNOS activity, indicating that tyrosine phosphorylation is required for eNOS activation. It has been observed, that 17-β-estradiol causes acute (within 5 minutes) activation of eNOS in cultured EC. This was not altered by the inhibition of gene transcription with actinomycin D. Thus, this process does not require the classical nuclear effects of the hormone. Acute mediation of eNOS by 17-β-estradiol and ER can cause the rapid activation of signaling pathways involving c-*src*-related tyrosine kinases and MAP kinase (18).

eNOS, which is localized in plasmalemmal caveolae, has been shown to interact with caveolin-1, the coat protein of caveolae. To identify the phosphorylated residues involved, immunoprecipitated ^{32}P-labeled eNOS was isolated from BAEC. Although a majority of the label was incorporated into phosphoserine, clearly detectable amounts of phosphotyrosine were also found. Identical results were described by using phosphotyrosine antibody 4G10 (the anti-phospho-tyrosine monoclonal antibody, which interacts with a broad range of phospho-tyrosine containing proteins). Thus, tyrosine phosphorylation is a regulatory mechanism, which influences eNOS activity, subcellular trafficking and interactions with other caveolin-interacting proteins. This shows the importance of protein-protein interactions in NO signal transduction (62).

Furthermore, in MCF-7 cells, synthesis of phosphatidylinositol and activation of phosphatidylinositol kinases can occur in response to 17-β-estradiol treatment (23).

3.2.2. Heat shock protein 90 (Hsp90)

Hsp90 has been implicated in the modulation of steroid receptor function. Hsp90 belongs to the heat shock family of stress proteins, which constitute 1% to 2% of the total soluble cytoplasmic proteins. This molecular chaperon actively participates in cytoprotection in the cardiovascular system. Hsp90 is a component of the reaction cycle involving newly synthesized proteins. Although their target proteins are steroid hormone receptors, the activity associated with their expression via the ER-Golgi pathway has not yet been very well determined (63). The interaction of the ER ligand-binding domain with Hsp90 has been characterized by studying a destabilizing mutation of the ligand-binding domain, which affects DNA binding and transactivation properties (64).

Besides phosphorylation, the activity of eNOS has been shown to be regulated by binding to heat shock protein90 (Hsp90). Involvement of Hsp90 in NO-dependent relaxation of intact blood vessels was determined in rat aorta. Ach-induced vasodilatation by NO was abrogated by geldanamycin, an ansamycin antibiotic, which inhibits tyrosine kinase (54). Immunoprecipitation of eNOS from bovine

microvascular endothelial cell (BMEC) lysates resulted in the coprecipitation of Hsp90. Moreover, immunoprecipitation of eNOS from rat aorta coprecipitated Hsp90, whereas precipitation of nNOS from rat cerebellum does not result in the association of these proteins. Vascular endothelial growth factor (VEGF), histamine, or growth factors, which increase the release of NO from EC, rapidly enhance the interaction between eNOS and Hsp90. This effect corresponded with a functional measurement of increased NO production. The rapid stimulus-dependent formation of the eNOS-Hsp90 heterocomplex indicates that this interaction precedes or occurs simultaneously with other signaling events, such as calcium mobilization or activation of other downstream effectors required for NO release.

Rapid Hsp90-eNOS association was produced by treatment of HUVEC with 17-β-estradiol. These endothelial cells secrete NO rapidly after stimulation with estrogen. NO release is due to activation of endothelial form of NOS. This activation appears to significantly reduce the calcium-calmodulin dependence of the enzyme (65). If we consider the analogy of shear stress-induced AKT activation to estrogen responses, this suggests that functional Hsp90-eNOS association is a critical downstream mediator of eNOS activation. (19). Nonetheless, the role of Hsp90 in estrogen-mediated activation of eNOS is still unclear at present (66).

3.2.3. Serine/threonine kinase AKT

AKT/PKB is a serine/threonine kinase (protein kinase-B) that requires a functional phosphatidylinositol 3-kinase to be stimulated by insulin and by other growth factors. It plays a role in protecting cells from apoptosis (67). The acute activation of AKT of a mutant murine estrogen receptor stimulates the phosphorylation of a protein, which regulates protein synthesis (PHAS-I) (68). AKT has been shown to be a downstream effector of estrogen- and insulin-like growth factor-1 (IGF-1)-dependent proliferation and survival in hormone-responsive MCF-7 breast carcinoma cells (23). NO production by EC in AKT-dependent manner was induced by simvastatin, which is a 3-hydroxyl-3-methyl coenzyme A reductase (HMG-CoA) inhibitor. In BAEC stimulated for 1 hour with simvastatin showed an increase in NO accumulation

without a detectable change in the concentration of eNOS protein (69). The AKT associated phosphorylation of eNOS and subsequent NO production were demonstrated after shear stress (25),(70) as well, as VEGF stimulation (24). The AKT pathway and its effects upon 17-β-estradiol stimulation and NO production through phosphorylation of eNOS, will be discussed in details in a separate chapter.

4. PI(3)K/AKT Signaling Pathway in 17-β-Estradiol Activation

4.1. Model of the serine/threonine kinase AKT activation

Originally identified as AKT8 retrovirus, its cellular homologue v-AKT was found to be a 57 kDa protein serine/threonine kinase. The N-terminus contains an Src homology 2 (SH2)-like domain, called a pleckstrin homology (PH) domain. Protein kinase B (PKB) is a downstream target of PI(3)K activation. PI(3)K phosphorylates phosphoinositides (PtdIns) at the 3-position of the inositol ring, generating 3'-phosphorylated phospholipids (PtdIns3*P*), phosphatidylinositol-3,4-bisphosphate (PtdIns(3,4)P_2) and phosphatidylinositol-3,4,5-triphosphate (PtdIns(3,4,5)P_3). PtdIns3*P* mediate the PI(3)K activation. PKB activity is induced in a PI(3)K-dependent manner. PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 specifically associate with PH domain. Recruitment to the plasma membrane is followed by a formation of PtdIns*P*. Transphosphorylation or autophosphorylation of PKB occurs at two residues, threonine 308 (Thr^{308}) within the P-loop of the protein kinase domain and serine 473 (Ser^{473}). An intermediate between PI(3)K and AKT is the protein kinase-1 (PDK1). PDK1 is an enzyme dependent on PtdIns lipids; it is therefore termed PtdIns(3,4,5)-dependent-PDK1. This enzyme specifically phosphorylates Thr^{308}. PDK1 is considered constitutively active and its function is instead regulated by subcellular localization. PDK2 is a protein kinase that targets PKB at Ser^{473}. This amino acid is autonomously regulated and is not an autophosphorylation site dependent on Thr^{308} phosphorylation. Transiently associating with PKB, PDK1 and PDK2 molecules are attracted to the plasma membrane by the basal levels of phosphorylated PtdIns molecules. These membrane-targeted PKB complexes gradually accumulate the necessary phosphorylation energy for its activation. Growth factors activate PKB via 85 kDa regulatory subunit of PI(3)K (67).

Protein kinase B (PKB), also known as c-AKT or RAC-PK (different from the small GTP-binding protein p21rac), is a proto-oncogene. A myristoylation causes relocation of the PKB from the cytosol to the plasma membrane. Plasma membrane localization

of PKB determines its constitutive activity. PKB contains a PH domain at its NH_2-terminus, which binds inositol lipids. In cells, the PKB is activated with mitogens stimulating in signaling pathways initiated by the phosphorylation of PI(3)K. A kinase activity that phosphorylates and activates PKB is revealed in the presence of PtdIns(3,4,5)P_3. PtdIns(3,4,5)P_3 has two functions in the activation of PKB – direct binding to the PH domain of PKB, which allows phosphorylation by the upstream kinase, and direct activation of the upstream kinase itself. PtdIns(3,4)P_2 activates the PKB directly, its effects on the upstream kinase is not confirmed. PI-3,4,5-trisphosphate (PIP3) and PI-3,4-bisphosphate (PIP2) are biologically relevant stereoisomers of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 respectively, which stimulate phosphorylation of PKB (71).

PTEN is a lipid phosphatase which dephosphorylates the PtdIns(3,4,5)P_3. By dephosphorylating PtdIns(3,4,5)P_3, PTEN acts in opposition to PI(3)K, the upstream activator of AKT. PTEN acts below PI(3)K but above AKT in the PI(3)K/AKT signaling pathway. When transfected into cells, levels of active phosphorylated AKT are diminished. PTEN through dephosphorylation of PtdIns(3,4,5)P_3 is considered one of a regulator of PI(3)K (72). The role of this tumor suppressor in the AKT activation is shown in a prostate cancer cell line lacking the PTEN. Loss of a functional PTEN results in high basal enzymatic activity of AKT. PTEN-lacking cells exhibit a constitutively elevated AKT (73).

AKT is serine/threonine kinase that requires a functional PI(3)K to be stimulated by growth factors. When directed to membranes by the addition of a *src* myristoylation sequence, AKT becomes constitutively active. A conditionally active version of AKT is constructed by fusing the AKT containing the myristoylation sequence to the HBD of a mutant murine ER that selectively binds 4-hydroxytamoxifen. The chimeric protein is expressed in NIH3T3 cells and is shown to be stimulated by hormone treatment. Stimulated AKT-estrogen receptor (myrAkt Δ4-129-ER) fusion protein induces the phosphorylation of a key regulatory site, Ser^{473}. The AKT-ER chimera must be present in a membrane compartment to be activated by PIP3 and PIP2 (68).

4.2. Effects of 17-β-estradiol on AKT/PKB signaling pathway

AKT is highly expressed in several human breast carcinoma cell lines and its activity in MCF-7 cells is modulated by 17-β-estradiol and IGF-1. Overexpression and activation of AKT in MCF-7 cells, sensitized by estrogen to the mitogenic effect of IGF-1, controls an anti-apoptotic pathway. Wortmannin blocks the growth response to 17-β-estradiol and IGF-1. Thus, AKT appears to be a common effector molecule downstream of PI(3)K that links the mitogenic effects of growth factors (23). In vascular smooth muscle cells (VSMC), PI(3)K is found to be a crucial upstream mediator for angiotensin II (Ang II)-induced AKT/PKB activation, inhibitable by the specific PI(3)K inhibitors Wortmannin and LY294002. AKT1/PKB activation by Ang II is redox-sensitive (74).

The predominant form of PI(3)K comprises p85α, an adapter/regulatory subunit of relative molecular mass of 85 kDa and p110, which is a catalytic subunit of molecular mass of 110kDa. PI(3)K catalyses the formation of lipid mediators through the recruitment of signaling molecules containing PIP3 and PIP2. The activation of AKT through phosphorylation of Thr^{308}/Ser^{473} mediates many of the downstream cellular effects of PI(3)K including activation of eNOS and cell survival pathways. For example, 17-β-estradiol stimulated eNOS activity in murine fibroblasts, transfected with ER-α and eNOS complementary DNAs, increases eightfold in wild- type but not in p85α –deficient ($p85\alpha^{-/-}$) fibroblasts. p85α dominant negative fibroblasts cotransfected with p85α cDNA lead to increase in 17-β-estradiol stimulated eNOS activity. Wild type fibroblasts co-transfected with a $p85\alpha^{-/-}$ cDNA decrease 17-β-estradiol stimulated eNOS activity. Increases in PIP-s time-dependently correlate with the ligand-dependent increases in ER-α-associated PI(3)K activity. These events are blocked by ICI 182,780 and Wortmannin, but not by the MAP kinase inhibitor PD 98059, or by the transcriptional inhibitor actinomycin D. To determine if 17-β-estradiol-stimulated eNOS activation is mediated by AKT, BAEC were transiently transfected with adenoviruses containing constitutively active (myr) and dominant-negative (dn) AKT mutants. Overexpression of myr-AKT causes substantial increase

in eNOS activity; dn-AKT completely abolishes 17-β-estradiol-stimulated eNOS activity (27).

The protein kinase AKT serves as a functional regulator of cell survival. The PI(3)K/AKT signaling pathway implication in endothelial NO regulation is shown on statins. Statins inhibit the activity of HMG-CoA reductase and are known for their lipid-lowering activity. AKT phosphorylation at amino acid residue, Ser^{473}, occurres in HUVEC within 30 minutes of statins treatment. PI(3)K/AKT signaling pathway inhibitors Wortmannin and LY294002 block statins-induced AKT (Ser^{473}) phosphorylation. The specificity of the kinase assay is demonstrated by infecting HUVEC cultures with an adenoviral vector encoding a dominant-negative form of AKT (Ad-dnAKT) that blockes increases in kinase activity (69).

AKT through PI(3)K mediation activates ER-α in the absence of estrogen. AKT ability to directly regulate ER-α activity is examined in transiently transfected ER-α-positive human breast cancer epithelial cell line MCF-7. Measured activity in cotransfected cells with constitutively active AKT expression vector results in 4.5- and 15-fold increases in activity in the absence or presence of estrogen, respectively. TMX has no effect. Therefore, AKT attributes estrogen-independent activity and further increases estrogen-stimulated activity of ER-α (75).

The effects of estrogen on cell cycle progression are shown on a series of pharmacological compounds that specifically inhibit signaling intermediates that have been implicated in estrogen actions. Treatment of starved MCF-7 cells with 20 nM of 17-β-estradiol stimulates cell cycle progression and results in 3-4-fold increase in the number of cells undergoing DNA synthesis. A disruption of the PI(3)K and MAPK cascades by specific inhibitors [Wortmannin, a PI(3)K inhibitor, PD098059, an inhibitor of MEK, the upstream activator of MAPK and antiestrogen ICI 182,780] prevent or reduce the ability of MCF-7 cells to enter the S-phase of the cell cycle in response to 17-β-estradiol. Interestingly, in this study, treatment with 17-β-estradiol has only a moderate effect on phosphorylated PKB levels. Nevertheless these data suggest that the activity of PI(3)K and MEK are implicated in the mitogenic effect of estrogen (76).

The fact, that estrogen is entering the AKT signaling pathway through PI(3)K is demonstrated in a study on its synergistic effects with IGF-1 in promoting cell proliferation of MCF-7. PI(3)K activity and AKT phosphorylation after IGF-1-receptor activation are enhanced by 17-β- estradiol treatment of the cells (77).

4.3. Estradiol activation of eNOS via the AKT/PKB pathway

The phosphorylation of eNOS is thought to regulate the activity of this enzyme in a Ca^{2+}-independent manner. As PI(3)K and the downstream serine/threonine kinase AKT/PKB are activated in EC by shear stress, the involvement of the PI(3)K/AKT pathway in the regulation of eNOS activity is investigated in HUVEC in response to shear stress. The rapid phosphorylation and activation of AKT assessed by western blotting is dependent on the degree of shear stress. The PI(3)K-inhibitor Wortmannin completely inhibits the induced increase in cyclic GMP levels. An overexpression of constitutively active AKT, created on Thr ^{308} and Ser ^{473}, results in 1.4 fold increase in intracellular cGMP, whereas overexpression of a dominant negative AKT mutant decreases basal cGMP levels by ~30%, indicating that eNOS can be activated by an AKT-dependent pathway. The role of AKT in eNOS phosphorylation is tested. In a model of bioassay, the Wortmannin completely abolishes the shear stress-induced NO release by vasoconstriction in an endothelium-intact coronary artery segments. eNOS is confirmed as a potential substrate for AKT phosphorylation by specific mutation of the two putative AKT-kinase sites on eNOS at residues Ser 633 and Ser 177, to aspartate (S633D, S1177D), which mimics continuous phosphorylation of the protein. In transfected HUVEC, there are no differences in measured eNOS activity by citrulline assay, between wild type and mutant eNOS. In contrast, overexpression of eNOS phosphorylation on Ser 1177 (Ser1177D, active mutant, aspartate), is prevented by replacement with inactive mutant alanine (S1177A). Finally, AKT-stimulated nitrite accumulation in COS cells transfected with wild type eNOS is abolished by mutating Ser 1177. Thus, Ser 1177 represents the primary phosphorylation site of eNOS in intact cells. Ser 1177 is an acceptor amino acid for

AKT-mediated phosphorylation. Serum-induced phosphorylation of eNOS is significantly decreased by PI(3)K inhibitor LY294002 or by co-transfection of a dominant-negative AKT-mutant. Shear stress-stimulated phosphorylation of eNOS is decreased by overexpression of a dominant-negative AKT mutant. These results indicate that *in vitro* eNOS is a target of AKT. Calcium-independence of eNOS phosphorylation is shown in cell lysates of wild type or mutant (S117D) eNOS transfected HUVEC in the presence of calcium. Results show that the stimulation of PI(3)K/AKT by shear stress or VEGF elicits the serine phosphorylation of eNOS, and thereby enhances enzyme activity in a Ca^{2+}-independent manner. The activation of eNOS by AKT is shown as an important physiological effect on apoptosis and proliferation (25).

The direct phosphorylation of eNOS on serine 1179 by the AKT (but not in mutant eNOS on S1179A) is shown on COS-7 cells, which do not express NOS but are co-transfected with eNOS and wild type AKT or kinase-inactive AKT. AKT induced eNOS phosphorylation, leads to the release of NO. AKT specifically phosphorylates the eNOS isoform; cotransfection of cells with the nNOS or iNOS does not increase NO production. In eNOS the presence of two putative AKT sites (serines 635 and 1179 in bovine eNOS and serines 633 and 1177 in human eNOS) is shown. Their respective importance for eNOS phosphorylation by AKT is examined in COS cells transfected with double mutant eNOS S635/1179A. Alanin (A) blockes the release of NO. When alanin is used to mutate S635 instead, this does not negatively influence the NO production. In contrast, serine 1179D (aspartate), which mimics the phosphorylation, when inserted into eNOS, augmentes the NO production induced by AKT. Cells transfected with both eNOS and AKT, when pre-treated with PI(3)K inhibitor Wortmannin, abolishe the AKT-induced increase in eNOS phosphorylation. The phosphorylation of eNOS by AKT does not require enhanced levels of calcium (24).

The involvement of AKT1/PKB signaling in eNOS phosphorylation is a novel mechanism, which applies to stimuli such as estrogen, VEGF or shear stress. The effects of an increase in eNOS serine phosphorylation are demonstrated using the okadaic acid, a specific serine/threonine phosphatase inhibitor. The eNOS serine

phosphorylation in the absence of the okadaic acid is associated with increase in NO production as determined by the accumulation of cyclic GMP. The eNOS phosphorylation is time-dependent and its maximum occurs after 5 minutes of tyrosine-residues phosphorylation following shear stress. Under Ca^{2+}-free conditions, the extent of the overall eNOS phosphorylation is lower than that observed in the presence of Ca^{2+}. A dominant-negative AKT mutant in transfected HUVEC inhibits the shear stress induced eNOS phosphorylation, as detected by phosho-serine eNOS specific antibody in western immunoblotting (70).

Simvastatin-induced AKT mediated phosphorylation of eNOS is examined by incubating HUVEC with inorganic ^{32}P. Endogenous eNOS phosphorylation is assessed by immunoprecipitation and visualized by autoradiography. Overexpression of dominant negative AKT in cells abrogates phosphorylation of eNOS. Alternatively, adenoviral infection with constitutively active AKT (myr-AKT) 24 hours before radiolabeling stimulates phosphorylated eNOS expression by autoradiography. In COS-7 cells, co-transfected with wild type eNOS and HA-AKT expression plasmid, simvastatin induces phosphate incorporation into eNOS. This process is inhibited when Ser 1179 in eNOS is mutated to an alanine residue S1179A (69).

Rapid eNOS activation by 17-β-estradiol, but not 17-α-estradiol, is evaluated by determination of NOS activity. HUVEC and TRLEC are treated with 17-β-estradiol in the range 10^{-10} – 10^{-7} M for 15 minutes. 17-β-estradiol induces the activation of eNOS in a dose-dependent manner. This effect, however, is not observed with supraphysiological concentration of 17-β-estradiol, 10^{-6} M (26).

5. Rationale

As shown by *F.Auricchio & A.Migliaccio* (1986), 17-β-estradiol, complexed with estrogen receptor, stimulates *in vitro* a 67-kDa tyrosine kinase (14;21;22). Similarly to the effects of the growth factors, they found that 17-β-estradiol directly stimulated tyrosine kinases and acutely induced protein phosphorylation by activating G-coupled protein receptors and tyrosine kinases in intact cells. (14;22) This mechanism of activation by 17-β-estradiol-ER complex involves the serine/threonine MAP kinase cascade.

Several studies have reported that different agonists responsible for proliferation could activate by phosphorylation other signaling pathways, such as the serine/threonine AKT/PKB pathway. As well, IGF-1 synergistically with 17-β-estradiol activate AKT in MCF-7 cells (23). The downstream AKT/PKB stimulation in EC, following an increase of NO production by eNOS phosphorylation, can also be induced by VEGF (24) and by shear stress (25;70). Shear stress induces NO release in a bioassay system with endothelium-intact pig epicardial artery segments. Release of NO from the endothelium can be elicited by vasoconstriction to increase shear stress at constant flow and the NO-mediated relaxant response to the shear stress is abolished by Wortmannin. Inhibition of PI(3)K prevents as well the shear stress-induced cGMP increase, and thereby the NO release, in HUVEC exposed to shear stress in the presence or absence of Wortmannin. Additionally, in VSMC, AKT/PKB via PI(3)K-dependent mechanisms, is stimulated by angiotensin and by exogenous H_2O_2 involving reactive oxygen species (ROS) in this mediation (74). Thus, ROS and the shear stress induced activation of eNOS involve the activation of the PI(3)K/AKT pathway (25).

6. Hypothesis

Activation of PI(3)K by tyrosine receptors can phosphorylate the serine threonine kinase AKT/PKB which then increases eNOS-phosphorylated activity. Since estrogen activates the membrane tyrosine kinase receptor, they could activate this pathway.

- **Therefore we examined whether the activation of PI(3)K by estrogen phosphorylates the AKT/PKB and increases the NO production and a vasodilatation.**

7. Materials and Methods

7.1. Materials

17-β-estradiol soluble in ethanol, water-soluble 17-β-estradiol (estradiol in cyclodextrin) and Wortmannin, were purchased from Sigma. ICI 182,780 and Tamoxifen were a kind gift from Dr Giguère, Dept of Oncology, Royal Victoria Hospital, MUHC. Anti e-NOS (N30020) antibody were purchased from Transduction Laboratories and anti phospho-eNOS (Ser1177), anti AKT and anti phospho-AKT (Ser 473) from Cell Signaling Technology. Chemiluminescence detection kit Lumi-Light^{Plus} Western Blotting Substrate was obtained from Boehringer Mannheim. We used the Tris-Glycine Sample Buffer (Novex™) and SeeBlue™ Pre-stained Standard (Invitrogen). L-[2,3- ^{3}H]-Arginine (specific activity 53.4 Ci/mmol) was purchased from NEN™ Life Science Products. All other reagents were purchased from Sigma.

7.2. Cell culture

We used cell cultures near confluence ≤ 90%, although studies from others have shown that 17-β-estradiol stimulated eNOS activity and NO production occurs in cells cultured to semi-confluence (78).

7.2.1. BAMEC

Immortalized BAMEC were plated ($4x10^4$ cells/plate) onto 100-mm plates in complete minimal essential medium (Eagle's) with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 100 u/ml penicillin / streptomycin and supplemented with 10% of heat inactivated fetal bovine serum (FBS). Cells were cultured at 37°C in a humidified atmosphere containing 5% (v/v) CO_2. Culture medium was changed every 2-3 days. Near confluent cells were detached by trypsinisation (0.05% trypsin) and passaged. 72-48 hours before stimulation the culture medium was replaced by complete MEM with 10% FBS, but without phenol red. Cultures were checked regularly under microscope for alterations in morphology, both during routine culture and prior to and after experimentations.

7.2.2. HUVEC

HUVEC were purchased from Clonetics BioWhittaker. The primary cells were routinely passaged in a standard cell culture medium as described (79) previously with slight modifications. These modifications consisted of using endothelial cell basal medium (EBM) supplemented with 20% FBS, 3 mg/ml bovine brain extract, 10 μg/ml human recombinant epidermal growth factor (hEGF), 50 mg/ml gentamicin, amphotericin-B 50 μg/ml, 2 mM glutamine, 5 U/ml heparin and 1 mg/ml

hydrocortisone in a water saturated atmosphere of 95% O_2 an 5% CO_2. Cell growth was routinely checked under a microscope and displayed the typical cobblestone appearance of endothelial cells (80). Subconfluent HUVEC monolayers grown in T-75 culture flasks, were studied within 5 passages.

For cell stimulation, in both BAMEC and HUVEC, we used only phenol-red free media and charcoal-stripped (CS) fetal bovine serum (FBS).
Charcoal-stripped FBS is an estrogen-depleted serum, prepared by adding PBS-rinsed Dextran-70 and charcoal at a 1:100 (wt/vol) and 1:10 (wt/vol) dilutions, respectively. The mixture was incubated at 55°C for 1 hr, centrifuged and subjected to filter sterilization. The content of $< 2.6 \times 10^{-11}$ M of estrogen residue is considered adequate for hormone removal by using described technique (81).

7.3. Cell stimulations

7.3.1. Time-dependence

At 90% of confluence, BAMEC were switched for 48 hours to a hormone-depleted growth medium prepared with phenol red-free Eagle's minimum essential medium (MEM) and supplemented with 10% charcoal-stripped (CS) FBS. Cells were starved 24 hours at 37°C before experiments in the above medium with 2% of charcoal-stripped FBS. Before stimulation, cells were rinsed once with phosphate-buffered saline (PBS). Control BAMEC cultures, incubated in parallel, received only medium or the vehicle in which the reagent solutions were prepared. Cells were incubated (with slight modifications) as described (14;22), for 1, 30 and 60 minutes with 17-β-estradiol (10 nM) at 37°C, or the vehicle (ethanol and DMSO) alone. The final concentration of ethanol in all cell cultures was ≤ 0.01%. The inhibitory effects to Wortmannin (50 μM) were assessed by exposing cells with 17-β-estradiol (10 nM) for 30 minutes. Parallel plates received similar dilutions of vehicles only, both DMSO

and ethanol in combination. Some cells were treated with the inhibitor Wortmannin alone or DMSO (as its vehicle) alone. All experiments were done in duplicates. Cell layers were harvested and lysed with ice-cold cell lysis buffer (CLB).

7.3.2. Estradiol-dose – response assay

48 hours prior to estradiol stimulation, HUVEC were cultured on 2%-gelatin-coated plates (2% gelatin solution in PBS), in phenol red-free medium M199 and in 10% of CS estrogen-depleted FBS. Nearly confluent HUVEC were starved overnight in 2% of CS-FBS. Cells were then stimulated with various concentrations of water-soluble 17-β-estradiol (17-β-estradiol in cyclodextrin, stocks prepared in PBS) ranging from 0.1 nM to 0.1 μM. Cells were conditioned with 17-β-estradiol during 60 minutes at 37°C. Some plates were exposed to specific estrogen receptor inhibitors, either ICI 182,780 (1 μM) or Tamoxifen 1 μM, or to the PI(3)K inhibitor Wortmannin (50 μM), 30 minutes prior to the stimulation with 17-β-estradiol 10 nM (18;23). Controls for the vehicle consisted of cells conditioned in medium only, in PBS-diluted cyclodextrin or in DMSO. Experiments were done in duplicates. Supernatants of stimulated cells were collected for NO_2^- analysis. Total cell lysate was prepared by scraping the cells in 800 μl of CLB and centrifuged at 10,000 rpm for 10 minutes. CLB contained (in mM) 50 HEPES (pH 7.5), 5.0 EDTA, $2H_2O$, 0.5% Triton X-100, 150 NaCl, 100 NaF, 10 Na-pyrophosphate, 100 Na_3VO_4 and protease inhibitors leupeptin 1 mg/ml aprotinin 2 mg/ml and pepstatin 1 mg/ml. Immunodetection by western analysis and NOS assays were determined in cell lysates. The protein measurements of all samples were performed with the Bradford assay.

7.4. Animals preparation

All procedures were approved by the Ethics Subcommittee of the University Animal Care Committee. We used female, oophorectomized Sprague-Dawley rats. Ovariectomy was performed by the supplier (Charles River Laboratories, St.

Constants, QC) in 8-week-old animals (200 - 225 gr) and shipped on the fifth day after surgery. A further 5 - 7 days of recovery were allowed in our Animal Center Department. Housing was provided for two rats per cage with access to water and food ad libitum, no special diet has been required. Animals were sacrificed by an intraperitonealy injection of dose 80 - 100 mg of sodium pentobarbital (MTC Pharmaceuticals). The aorta was quickly harvested and put into ice-cold Krebs-bicarbonate solution containing (in mM): 118.4 NaCl, 25.0 $NaHCO_3$, 4.7 KCl, 1.1 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 5.6 Glucose.

7.5. Aorta rings bioassay

We studied an acute vasodilatory effects of estrogen on induced NO release in isolated rat aortic segments. Therefore, our primary objectives were:

1. To determine the NO release from endothelium of 17-β-estradiol-incubated aortics rings compare to the rings incubated without 17-β-estradiol, and 2. To determine the effect of AKT/PKB inhibitor Wortmannin on 17-β-estradiol-induced NO increase (if it was a case).

The vessel was cleaned of adventitial adipose and connective tissue and cut into ~3 mm segments. Aortic rings were mounted horizontally between two stainless steel parallel hooks for the measurement of isometric tension (a force transducer Grass-FT03) and were suspended in organ bath (18 ml) filled with 37°C warm and continuously oxygenated (95% O_2 / 5% CO_2) Krebs-bicarbonate (pH 7.4) buffer (82). Vessels were equilibrated about 40 minutes before they reached optimal resting tension. The resting tension is the optimal point of the vessel's cross-sectional length-tension relationship. To define a resting tension, vessels are progressively stretched until the contraction is maximal to 60 mM of KCl solution applied in the organ bath bathing the vessel ring (83). As determined from our previous experiments, the resting tension of the rat aorta is 2 gr.

For the first condition, resting vessels were preconstricted with α_1-adrenergic agonist phenylephrine, in the concentration 10^{-7} M. The integrity of the endothelium was

assessed by increasing Ach concentrations in a range of 0.1 nM – 100 μM (84). Magnitude of these dilations was expressed as percentage of maximum contraction provoked by 10^{-7} M Phe. After 40-minutes of preincubation with 10^{-7} M 17-β-estradiol dissolved in ethanol (≤0.01%), which was added to an organ bath, the aortic rings were preconstricted again with Phe 10^{-7} M and exposed to the same vasodilator concentrations as in previous step. An Ach relaxation dose-response curve was created again. After washout, the equilibrated vessels were preconstricted again and increasing doses (10^{-9} – 10^{-5} M) of 17-β-estradiol were added to verify its direct dilatory effects.

In the second condition, we tested the inhibition of estradiol-induced nitric oxide production by AKT/PKB inhibitor Wortmannin. The vessels were preincubated for 10 minutes with 10^{-7} M of Wortmannin, before the first Phe preconstriction. Vasodilatory dose-response curve with increasing concentrations of Ach was made. Vessels were then preincubated with 17-β-estradiol, preconstricted with Phe and endothelium-related vasodilatation was verified with Ach in increasing concentrations (10^{-10} – 10^{-5} M). Wortmannin efficiently abolished the 17-β-estradiol-stimulated NO increase and blocked subsequent vascular relaxation previously seen in aortic segments incubated with 17-β-estradiol.

In our preliminary experiments, a cumulative dose-response of Phe (10^{-10} – 10^{-4} M (85)) was created to measure sensitivity and determine the dose that would give a 70% constriction for each individual arterial ring. Since the vessel constriction is maximal and reaches plateau with Phe 10^{-4} – 10^{-3} M, the concentration of Phe 10^{-7} M is a concentration, which produces 70% of the maximum contraction. This pharmacological dose was used to constrict the aortic ring to achieve a baseline from which were measured subsequent relaxation responses (50).

7.6. Determination of HUVEC NO_2^- release

Cells were stimulated with 17-β-estradiol for 60 minutes at 37°C. Supernatants of conditioned cells were collected in separated tubes for nitrite measurement. Tubes were stored at -80°C before measurements of nitrite.

Deproteinized samples (zinc-sulphate 30%, 1:20 v/v) were passed through a copperized cadmium reduction column in which NO_{3-} was reduced to NO_{2-}, which reacts with Griess reagent (1% sulfonamide in 5% H_3PO_4, 0.1% N-naphtylethyl-ethylendiamine dihydrochloride in deionized distilled water) to form a purple azo dye. 300 µl of media supernatant was added the equal amount of Griess reagent. Absorbance at 540 nm was detected by spectrophotometer against the $NaNO_2$ standard curve (78;86;87). The blank absorbance obtained in wells which were treated and incubated identically, but which lacked cells, was subtracted. Since dimethyl sulfoxide (final concentration of 0.01% in the culture medium), the solvent of Wortmannin, is known to cause a high background in the incubation medium, the individual data were corrected for this nonspecific effect (88). The detection limit of the assay was 2.5 µM.

7.7. NOS activity in BAMEC

eNOS activity was determined as described previously (87) in total cell lysates of BAMEC (30). Lysate protein (80 - 90 µg) was incubated for 10 minutes at 37°C with 100 µl of reaction buffer (pH 7.2) containing (in mM): 50 potassium-phosphate buffer, 1 mg/ml bovine serum albumin (BSA), 0.2 tetrahydrobiopterin (BH_4), 1.2 magnesium chloride ($MgCl_2$), 0.6 flavin adenine dinucleotide (FAD), 0.2 flavin adenine mononucleotide (FMN), 6 L-Valine, 1.0 reduced nicotinamide adenine dinucleotide phosphate (NADPH), 10 µg/ml calmodulin (CaM), 0.75 calcium chloride ($CaCl_2$) and 25 µl of 120 µM stock L-[2,3-^{3}H]-Arginine (150 - 200 counts per min/pM). Parallel incubations were performed in the presence of the specific NOS inhibitor 1.5 mM *N*-nitro-L-arginine-methyl ester (L-NAME). Incubations were terminated by adding 500 µl of ice-cold stop buffer [50 mM Hepes, 12 mM ethylenediaminetetraacetic acid (EDTA), pH 5.5]. L-[^{3}H]-citrulline was separated from the mixture by passing the mixture through a column of equilibrated Dowex-50W strong acid cation exchanger (50WX8-400, Na^+ form). The eluate (1.6 ml) was

collected into scintillation liquid and the NOS activity was measured by counting the radioactivity in the eluate.

The positive control of NOS activity measurement was prepared from freshly isolated rat cerebellums from 300 gr Sprague-Dawley male rats. Tissue supernatant was obtained by quick homogenization of pooled cerebellums in ice-cold homogenization buffer and preparation was centrifuged at 10,000 rpm/10 minutes at 4°C. Protein was determined in the supernatant by the Bradford method. Aliquots were stored at –80°C.

7.8. Western immunoblotting

Phosphorylation of AKT and eNOS was assessed in cell cultures conditioned with increasing concentration (0.1 nM – 10 μM) of 17-β-estradiol or in cells treated in time-course (1, 30, 60 minutes). Lysed cells were centrifuged at 10,000 rpm in a microcentrifuge for 10 minutes at 4°C. Protein supernatant (25 - 40 μg) were separated using 8 - 10% SDS-PAGE (Mini Protean II, Bio Rad) and transferred using a semi-dry protein transfer apparatus (BioRad) to PVDF membranes. Membranes were blocked 1 hour at room temperature and incubated overnight at 4°C with primary rabbit polyclonal phospho-specific antibodies (Abs). Membranes were washed 4 times within 1 hour in Tris-buffered saline containing 0.1% (v/v) Tween (TBS-T) (pH 7.5). After 1-hour incubation at room temperature with secondary horseradish peroxidase-conjugated (HRP) goat anti-rabbit Abs, phosphorylated forms of proteins were detected by enhanced chemiluminescence using substrate Lumi-Light^{PLUS} , followed by autoradiography. Immunoblots probed for both, phospho-AKT and phospho-eNOS were stripped (in 0.2 N NaOH for 15 minutes at RT and washed twice for 10 minutes in distilled water), blocked again and reprobed overnight at 4°C with primary total AKT or total eNOS, respectively, followed by one hour incubation at room temperature with secondary horseradish peroxidase-conjugated Abs, to confirm equal loading of the gel. All blots were scanned and subjected to measurement of optical density of bands in SigmaGel (Jandel Scientific). The signal intensity was expressed in absolute values. A ratio of the signal intensity of the

phospho-serine blots vs. total-protein blots was determined to normalize for differences in loading (25). The value of ratio for controls was arbitrary set at 1. Values of all other bands were defined as fold of increase of controls.

7.8.1. Immunodetection of phosphorylated AKT / PKB

As described, (23), (74) phosphorylated AKT was detected with primary rabbit polyclonal specific phospho-AKT (Ser 473) antibody (Ab) diluted 1:1500. Secondary anti-rabbit HRP-Ab was diluted 1:4000. For a negative control, a nonphosphorylated AKT cell extracts were used from untreated NIH 3T3 cells. As positive control, phosphorylated AKT cell extracts were used from PDGF (50 ng/ml for 20 minutes) treated NIH 3T3 cells. Phospho-AKT immunoblots were stripped and blotted (25) for total AKT with polyclonal Ab diluted 1:1500. Secondary anti-rabbit HRP-Ab was diluted 1:4000.

7.8.2. Immunodetection of phosphorylated eNOS

Phosphorylation of eNOS on serine 1177 as a potential phosphorylation site in human eNOS (24) was assessed by using polyclonal phospho-specific primary Ab diluted 1:1000, directed against serine 1177. Secondary anti-rabbit HRP-Ab was diluted 1:3000. To determine the total eNOS, stripped and re-blocked (TBS-T solution with 5% of non-fat dry milk) membranes were reprobed (65) with monoclonal primary anti-eNOS Ab (1:1250) and secondary anti-mouse Ab (1:1500).

7.9. Statistical Analysis

Data are expressed as mean ± SEM from 3 to 5 individual experiments. Statistical analysis between two groups was performed using Student's *t* - test, comparisons between multiple groups were made by one-way ANOVA with a post-hoc Bonferroni test or by two-way repeated measures ANOVA followed by Student-Newmann-Keuls test. Probability values were considered significant at $P < 0.05$.

8. Results

8.1. Phosphorylation of serine/threonine protein kinase AKT by 17-β-estradiol is time-dependent.

It has been shown that serine-threonine cascade could be activated in EC in response to shear stress. (25) The BAMEC were treated with 10^{-8} M of 17-β-estradiol for 1, 30 and 60 minutes. Phosphorylation of AKT (protein kinase B) shown in **Figure 1A, upper panel**, occured as early as 1 minute (*lane 6*) after treatment with 17-β-estradiol and increased ≈2.5 fold compared to controls. The intensity of AKT phosphorylation increased in 30 minutes (*lane 7*) and was maintained for 60 minutes (*lane 8*). There was no activity in control cells in media, nor in cells treated with solvents (ethanol 0.01% nor DMSO) (*lanes 1– 4*). The PI3-K inhibitor Wortmannin, applied on cells for 60 minutes at a concentration of $5x10^{-6}$ M with 10 nM of 17-β-estradiol, completely antagonized the AKT phosphorylation (*lane 9*). No signal was detected on the western blot with Wortmannin alone (*lane 10*). There were no differences in the non-phosphorylated AKT. (**Figure 1A, lower panel**). As seen in the graph (**Figure 1B**), 17-β-estradiol stimulated AKT phosphorylation in a time-dependent manner. Phosphorylation of AKT peaked at 30 minutes and sustained phosphorylation lasted for up to 60 minutes. The graph represents data of 2 individual experiments.

8.2. 17-β-estradiol dose-response of AKT phosphorylation.

The phosphorylation of AKT on serine 473, is shown in HUVEC treated for 60 minutes with increasing concentration of 17-β-estradiol in the range of 10^{-10} - 10^{-7} M. This effect was 17-β-estradiol dose-dependent (**Figure 2A, upper panel**). Treatment with estradiol 10^{-10} M did not increase AKT phosphorylation (*lane 3*) if compared to vehicle control (*lane 9*). Phosphorylation was increased by ≈1.3 fold with 17-β-estradiol 10^{-9} M (*lane 4*). Maximal phosphorylation was observed in treatment with 17-β-estradiol 10^{-8} M (*lane 5*), ≈1.8 fold. No further increase of AKT phosphorylation was observed by exposing cells to the concentration of 17-β-estradiol 10^{-7} M (confirmed in 3 other immunoblots, not shown). Wortmannin 50 μM inhibited the effect of 17-β-estradiol 10^{-8} M on AKT phosphorylation (*lane 8*). The estrogen receptor inhibitor ICI 182,780 0.01 μM did partially block this process (*lane 7*). Immunoblotting with total AKT antibody did not demonstrate 17-β-estradiol dose-dependence; total AKT is well expressed in all cell stimulations (*lanes 1 - 8*) (**Figure 2A, lower panel**). The bar graph (**Figure 2B**) shows the ratio of the signal intensity of the phospho-AKT blot vs. the total-AKT blot. In another set of experiments in HUVEC, the time-dependence of cells stimulated with increasing concentrations of 17-β-estradiol was measured. As shown in **Figure 2C, upper panel**, in *lane 7*, Tamoxifen 1 μM, an estrogen receptor inhibitor, abrogated the AKT phosphorylation by 17-β-estradiol 10^{-8} M. Phosphorylation of AKT peaked at 30 minutes of 17-β-estradiol 10^{-8} M stimulation (*lane 5*), and decreased by 60 minutes of treatment (*lane 6*). Shown data are representative for four separate experiments.

8.3. Phosphorylation of eNOS by 17-β-estradiol.

Figure 3A, upper panel, shows the effect of 17-β-estradiol on phosphorylation of eNOS in HUVEC. We examined whether 17-β-estradiol, through membrane bound estrogen receptor and downstream recruitment of AKT, mediates phosphorylation of eNOS on serine 1177 and if this induction is 17-β-estradiol dose-dependent. eNOS

phosphorylation in HUVEC was assayed in cells treated 60 minutes with 17-β-estradiol (concentrations 10^{-10} - 10^{-7} M). Immunoblot was probed with specific phospho-eNOS (ser 1177) antibody. 17-β-estradiol 10^{-10} M increased eNOS activation ≈1.5 fold (*lane 2*), whereas 17-β-estradiol 10^{-9} M intensified this process ≈2.1 (*lane 3*). Maximal eNOS phosphorylation occured at 17-β-estradiol 10^{-8} M, ≈2.3 fold (*lanes 4-5*). Further increasing the concentration of 17-β-estradiol to 10^{-7} M, did not change the eNOS phosphorylation (*lane 6*). To test the AKT signaling pathway implication in the subsequent eNOS phosphorylation, cells were exposed to the AKT specific inhibitor Wortmannin 50 μM. The phosphorylation of eNOS associated with 17-β-estradiol 10^{-8} M was completely blocked (*lane 8*). The role of membrane estrogen receptor in 17-β-estradiol mediated eNOS phosphorylation, was determined by treatment of HUVEC with the specific estrogen receptor inhibitor ICI 182,780 0.01 μM combined with 17-β-estradiol 10^{-8} M (*lane 7*). ER inhibitor almost completely prevented eNOS activation and yielded only a weak signal on the immunoblot probed with phospho-eNOS (Ser 1177) antibody. Total eNOS protein expression was equal in all treatments, independent of either, 17-β-estradiol concentration or in the presence of either AKT specific estrogen receptor inhibitors (**Figure 3A, lower panel**). The increase in phosphorylation of eNOS by stimulating with physiological concentration of 17-β-estradiol was statistically significant. The column graph (**Figure 3B**) expresses the densitometric ratio of the phosphoserine blot vs. the eNOS blot (24;70). The immunoblot is representative of 5 experiments, (mean ± SEM, $P < 0.05$).

8.4. 17-β-estradiol mediates acute increase of eNOS activity.

Since we found by immunoblotting that the effect of 17-β-estradiol on plasma membrane through the AKT signaling cascade resulted in eNOS phosphorylation, we tested next if this process could enhance the eNOS enzymatic activity in EC. The eNOS activity after L-NAME inhibition was assayed in cell lysates from control (vehicle) and 17-β-estradiol 10^{-8} M treated BAMEC. Cells were stimulated in

different periods of time (1, 30 and 60 minutes) and in the presence of the AKT inhibitor Wortmannin 50 μM. Exposure of cells to 17-β-estradiol 10^{-8} M for 60 minutes increased eNOS activity ≈2.6 fold (corresponds to 102 pmol / mg / min) (**Figure 4**). Wortmannin blocked the 17-β-estradiol mediated eNOS activity to the level of vehicle control (corresponds to 40 pmol / mg / min). Thus, we tested the 17-β-estradiol-induced increase in eNOS activity via the PI(3)K/AKT signaling pathway. Time course of the effect of 17-β-estradiol 10^{-8} M in the presence of eNOS specific inhibitor L-NAME did not reveal any changes. Measured eNOS activity sustained the level detectable in quiescent cells. Physiological concentrations of 17-β-estradiol (10^{-8} M) caused eNOS activation in EC within the first minute of exposure to the hormone. This observation is consistent with the non-genomic character of the response to 17-β-estradiol treatment in the vasculature and in the EC (17;18).

8.5. 17-β-estradiol stimulates nitrite oxide release.

Next we determined if consequently there is a higher nitrite oxide production. The nitrite accumulation in cell supernatant was measured by Griess non-enzymatic reduction method spectrophotometrically. **Figure 5** shows the change in nitrite in the supernatant of human endothelial cells treated with 17-β-estradiol by dose-dependent manner (in the range of 0.1 - 100 nM). Basal nitrite accumulation in control (untreated) cells was 5.99 μM/ 10^5 cells ± 1.0 (*column 1*). Similar values were obtained of cells treated with vehicle alone (*column 2*) or cells treated with 17-β-estradiol 10^{-10} M (6.44 ± 2.1 and 5.69 ± 0.6 μM/10^5 cells respectively) (*column 3*). 17-β-estradiol 10^{-9} M and 17-β-estradiol 10^{-8} M increased nitrite release to 15.99 ± 4.2 and 12.58 ± 0.8 μM/10^5 cells respectively (*columns 4 –5*). The increase of 17-β-estradiol 10^{-9} M was statistically significant (mean ± SEM, n = 5, $P < 0.05$). The increase in 17-β-estradiol to 10^{-7} M (*column 6*) did not result in higher NO production (10.86 ± 5.6 μM/10^5 cells). The PI(3)K inhibitor Wortmannin 50 μM, and both ERs inhibitors, ICI 182,780 0.01 μM and Tamoxifen 1 μM (*columns 7 –9*), completely

abrogated NO generation (4.34 ± 1.2, 6.62 ± 1.1, 4.3 $\mu M/10^5$ cells respectively) in cells treated concomitantly with 17-β-estradiol 10^{-8} M. The individual data were corrected for the nonspecific effect of solvents. Results represent means ± SEM of 3 to 5 individual experiments.

8.6. Endothelium-dependent vasorelaxation induced by rapid effects of 17-β-estradiol.

The magnitude of endothelium-dependent vasorelaxation in Phe-preconstricted rat aorta rings mediated by cumulative concentrations of Ach (10^{-10} – 10^{-5} M) was EC_{50} = 0.15 x 10^{-7} M (**Figure 6A**, ♦*dotted line*). 40-minutes of incubation with 17-β-estradiol (10^{-8} M) induced a left-shift of the Ach-mediated concentration-dependent vasorelaxation curve in Phe-preconstricted vessels. The magnitude of vasodilatation in 17-β-estradiol preincubated rat aortic rings was EC_{50} = 0.7x10^{-8} M (■ *full line*). These vasodilator responses to Ach after 17-β-estradiol incubation were significantly greater than the responses to the same concentrations of Ach before 17-β-estradiol exposure ($P < 0.05$, n = 6). 17-β-estradiol treatment resulted in an intensive NO production from endothelium of rat aorta, which caused a leftward shift of the Ach-triggered dose-response curve. The vasorelaxant effect of 17-ß-estradiol was abolished by pre-treatment of rat aortic segments with PI(3)K antagonist Wortmannin (**Figure 6B**). There were no differences in the amount of NO mediated Ach-vasorelaxation between aortic rings incubated with Wortmannin alone or after concomitant incubation with 17-β-estradiol. Ach–mediated cumulative concentration-dependent vasorelaxant curves were similar in both, aortic rings incubated with Wortmannin alone (▲*broken line*) or after co-incubation of Wortmannin and 17-β-estradiol (■ *full line*). As a result, the pattern of both Ach-triggered relaxation curves, the one with inhibitor and the other with the inhibitor plus the NO-stimulating agonist 17-β-estradiol, remained unchanged. These data indicate that in endothelium, 17-ß-estradiol via the activated AKT/PKB pathway subsequently increases NO production. Data represent means ± SEM of 6 individual experiments.

9. Final Conclusion and Summary

9.1. Main Findings

- Estradiol treatment resulted in phosphorylation of AKT/PKB in both, bovine and human endothelial cells
- Estradiol treatment also resulted in phosphorylation of eNOS in human EC
- eNOS phosphorylation was associated with increased NO production
- Estradiol treatment enhanced Ach mediated relaxation of isolated aorta from ovariectomized rat and this was blocked by a PI(3)K inhibitor

10. Discussion

The central findings of this study are that estrogen treatment leads to rapid phosphorylation of eNOS in EC and increases NO production via the phosphorylation of AKT through the PI(3)K activation. This non-genomic action of estrogen requires the presence of ERs but it is independent of gene transcription (27;30;89). Classically, estrogen effects are mediated through genomic mechanisms that involve the diffusion of estrogen across the plasma membrane and activation of specific intracellular receptors by modifying gene expression (8). However, there are also important rapid, nongenomic effects of estrogen and other related steroid hormones in a variety of tissues, such as vasculature, brain and bone (8;18;29). Accumulating evidence indicates that steroid hormones act via the classical (type-1) steroid receptor, but also by binding to non-genomic sites (29). Rapid response to acute 17-β-estradiol treatment is unaffected by the inhibition of gene transcription with actinomycin D (30;90). Non-genomic responses to estrogen occur within minutes. Thus, in contrast with classic estrogen receptor-mediated responses, such rapid events do not require gene activation. The pure estrogen receptor antagonist ICI 182,780 completely blocks many of these rapid signaling events. Most probably a membrane-located receptor,

which acts as a rapid, transmembrane signaling molecule, mediates the non-genomic responses (91).

The precise mechanism by which estrogen increases the basal NO release from intact endothelium is still not completely understood. The physiological significance of the AKT/PKB signaling pathway in NO bioavailability was shown in several models. For example, AKT/PKB signaling promoted angiogenesis in ischemic limbs of normocholesterolemic rabbits by statins (69).

10.1. Experimental approach and its limitations.

10.1.1. Choice of animal model

For the experiment on aorta rings, we purchased female Sprague-Dawley rats (200-225 gr), which were already oophorectomized at 8-weeks of age. We chose rather oophorectomized rats than intact animals, because of the controversy on rat vascular reactivity, which is present in the literature. In a previous study on non-oophorectomized animals the vascular responsiveness of rat mesentery artery to capsaicin-sensitive sensory nerve stimulation caused by nicotine was not affected by the estrous cycle (92). Contradictory to this, another study showed a slightly smaller response to phenylephrine in the aorta from cycling rats in estrus compared to metestrus (93). The vascular contractility of coronary arteries was substantially lower in intact female and oophorectomized but estrogen supplemented rats compared to the contractility of arteries exposed to little or no circulating estrogen in male rats and oophorectomized rats. This was thought to be due to the enhanced NO release in intact female rats as well as ovariectomized estrogen-supplemented rats (42).

The rat was chosen as the animal model in the present study because the hormonal status of the animal can be altered relatively easily by ovariectomy. Oophorectomized animals are suitable for a study of estrogen effects on vasculature, because

ovariectomy eliminates differencesthat exist within the estrus cycle of intact female rats.

Oophorectomy elicits a significant reduction of plasma estrogen levels. The concentration of 17β-estradiol in the plasma of cycling rats is 144.7 pg/ml compared to 5.3 pg/ml in the plasma of oophorectomized rats (94).

Because it is also known from studies with oophorectomized rats, that serum estrogen concentrations even after 3 weeks of ovarectomy, remained significantly lower than of intact animals (94;95), we considered one week of recovery after oophorectomy sufficient to eliminate the estrogen which was produced by the ovaries, from the vascular circulation of rat (96). The use of estrogen-withdrawal animals allowed us to restore estrogen to a fixed level, so that we could compare response of isolated aorta rings from different animals.

To avoid time effect between oophorectomy and the day of the experiment, we ordered 6 animals per one set of experiments. All underwent an oophorectomy in the same day. The experiment with only one animal was performed daily, given the number of available organ chambers, so that there was only a delay of 6 days between the first and the last experiment of the same set of animals. Vascular responsiveness of delayed oophorectomized rats could be influenced from estrogens produced endogenously by the other sources or as a consequence of the greater tissue mass (97).

10.1.2. Aorta segments bioassay

In our experiment, we used aorta from rat. Protocols with rat aorta rings are well established and there is already data on the effect of estrogen in this model (34;85;98). It was particularly suitable for the experiment of isometric tension, adopted as reliable in previous experiments for expressing endothelium-derived accumulation of NO from vasculature. The size of rat aorta allows easy handling and a reproducibility of results.

We used Ach, a muscarinic receptor agonist that mediates the endothelium-derived NO (49). It produces endothelium-dependent relaxation of endothelium-intact human epicardial coronary arteries (32) as well as rat aortic rings (50;85). It has been reported that the relaxation of isolated aorta and other arteries induced by Ach, depends on the presence of endothelial cells in the vessels. After the removal of these cells either mechanically, enzymatically or with atherosclerotic plaques (32), Ach no longer induces relaxation (85;98).

Therefore, the basic condition in our experiment was to preserve integrity of rat aorta endothelium. Aorta was handled very carefully in order not to damage the vascular lining. Precontracted, endothelium-intact aortic rings relaxed in a dose-dependent manner in response to increasing concentrations of Ach (10^{-10} – 10^{-6} M). The curves that we obtained reflect the amount of basal release of NO from intact vascular endothelium. In order to determine if acute administration of 17-β-estradiol increases release of NO, the same aortic segments were incubated with 17-β-estradiol and NO release was triggered in preconstricted aortic rings with the same concentrations of the mediator Ach. We compared the differences between basal productions of NO from aortic endothelium and 17-β-estradiol-stimulated expressed as 50% (EC_{50}) of maximum Ach-mediated dilation for each single ring. We demonstrated, that the same concentration of Ach in untreated rings mediated lower NO production than in 17-β-estradiol-treated aortic rings. Vasorelaxation dose-response curves shifted leftward. There was a 4.5-fold increase in NO production of 17-β-estradiol-acutely stimulated aortic rings compared to untreated segments of vessels. The difference in magnitude of vasorelaxation between treated and untreated aortic segments was statistically significant ($P<0.05$) for the group of 6 rat aortas.

If our hypothesis is true, that acute 17-β-estradiol stimulating effect on NO production is due to activated AKT/PKB pathway, then Wortmannin, a PI(3)K inhibitor, must abrogate the increase in NO from Wortmannin-effected aortic endothelium. Therefore, to assess the effect of Wortmannin on basal NO release, we measured Ach-mediated vasorelaxation in aortic segments incubated only with Wortmannin and we determined whether Wortmannin inhibits the 17-β-estradiol

mediated leftward shift. Indeed, incubation with Wortmannin prevented the 17-β-estradiol- increase in Ach-mediated vasorelaxation.

At the end of the experiment we obtained 17-β-estradiol dose-response curves to test its direct effects on the vasculature. In 2 assays, increasing dilation was observed mainly with 17-β-estradiol in supraphysiological concentrations (10^{-6} – 10^{-5} M), in 2 other assays there were no changes in 17-β-estradiol vasorelaxation curve (data not shown). A direct 17-β-estradiol-vasodilatory effect was already shown on precontracted intact female rat aorta endothelium with 10^{-9} M of 17-β-estradiol (99). Studies of 17-β-estradiol on de-endothelized rat arteries suggest that its effect is possibly on VSMC. For example, pharmacological doses of 17-β-estradiol dilate denuded male rat tail artery (34) as well as dilate preconstricted de-endothelized female rat aorta (100). Because in our study integrity of endothelium was repeatedly confirmed by Ach-mediated vasodilatation, the role of direct effects of 17-β-estradiol on the vascular wall cannot be assessed.

In our protocol, vessels were preconstricted on repeat occasion with 10^{-7} M of phenylephrine and dilated with increasing concentrations of Ach. The repeated stress could have "masked" the effect, i.e. it wouldn't work. In other words, the Ach-triggered vasorelaxation would be due to the passive relaxation rather than to the NO overproduction by 17-β-estradiol. To avoid this possibility, we incubated vessels for only 30-minutes with 17-β-estradiol, or 17-β-estradiol plus Wortmannin under controlled equilibrated resting tension. If differences within each protocol were more than 25% between first and second preconstriction with Phe, these segments were discarded due to the possibility of failure of the contractile response. We considered incubating aortic segments with 17-β-estradiol in the first part of protocol, so that we could reverse the order between the first and second part of the protocol. We did not proceed in this manner because estrogen would most likely had residual effects on the vessels. Another approach would have been to perform a side-by-side protocol on two aortic segments. One of them could have been preincubation with 17-β-estradiol in the incubation period before the second Phe preconstriction. The other aortic segment assayed in separated organ chamber could have been incubated without 17-β-estradiol in both steps of the protocol. The magnitude of Ach vasorelaxant effects then would

be compared between two aortic segments. The major disadvantage of that type of protocol is that it requires two individual segments. Even if these were from the same animal, rings would have to be cut from different topological sites of the aorta, which could result in differences in vascular responsiveness. Not negligible, would also be the requirement of doubling the amount of necessary experiments.

We did not perform a 17-β-estradiol relaxation-response curve in Wortmannin-pretreated aortic segments, because the inhibitory effects of Wortmannin on activity of PI(3)K are irreversible (101).

Incubation of vessels with the vehicle only (ethanol 0.01%) instead of active substance (17-β-estradiol), was done in preliminary experiments. No dilatory effect of solvent was observed on control segments.

10.1.3. Cell cultures

We used two different cell lines: bovine adrenal medulla endothelial cells and human umbilical vein endothelial cells.

10.1.4. BAMEC

BAMEC are immortalized cell line and for this reason may lack certain functions. On the other hand, such cell culture depends much less on a high-serum content in the milieu, and will have less apoptotic cells in the period of serum-depletion. In our preliminary experiments, we did not find expression of eNOS protein in bovine endothelial cells. However, we subsequently identified eNOS activity in BAMEC. Because phopshorylation of AKT was one of our goals, we first confirmed by western analysis that AKT was present. Because we were looking for a receptor-mediated system, we verified by RT-PCR that there was expression of ER-α and even more strongly, ER-β.

10.1.5. HUVEC

We also used HUVEC. These endothelial cells have preserved the specialized endothelial cell activities, for example NO production in response to vasoactive proinflammatory cytokines (102). Because the target of our study was the stimulating effect of 17-β-estradiol on NO production, as well as eNOS phosphorylation, we have chosen HUVEC where eNOS is expressed constitutively. Since we studied the effects of 17-β-estradiol as an agonist, and HUVEC are known to express estrogen receptor (ER), the choice of the primary human endothelial cell culture seems to be more convenient as the model of the 17-β-estradiol induction than the use of the permanently established HUVEC-sarcoma fusion cell line EA.hy926 (19), where ER has not yet been cloned or characterized (66). Also, we confirmed a presence of ERs in preliminary study by RT-PCR. We found only weak amount of ER-α and abundance of ER-β.

Subconfluent HUVEC monolayers were studied within 5 passages. In experiments on HUVEC performed on passage 7, detectability of eNOS by western immunoblotting was very weak, as well as was the nitrite determination in supernatant. This experience is consistent with the fact that the tyrosine phosphorylation of eNOS was only observed by groups using primary cultures or low passages of EC. The effect of 17-β-estradiol on the activity of eNOS in both, human and bovine EC, decreased gradually when the culture passage was more than 5^{th} passage. It is apparent that the intracellular mechanisms involved in regulating the phosphorylation of eNOS are rapidly lost in culture (62;103). The existence of estrogen receptor in these endothelial cells markedly decreased as well after the 5^{th} passage (78). Generally, HUVEC are used most often in primary cell culture or between passages 2 – 5 (78-80;88;104).

Phenol red

For culturing of both cell lines, we used only media without phenol red to avoid potential estrogen-mimicking effects of this compound. Phenol red, a pH indicator in most tissue culture media, has been said to act as a weak estrogen in some cell lines

(105). However, it was shown in a more recent study, that phenol red had no estrogen-simulating effects in HUVEC (106). Because we used 17-β-estradiol as a stimulator, to avoid this potential confound, 72-48 hours before conditioning of cells, we strictly used only culture medium without phenol red (14;27;30;86).

Serum deprivation of HUVEC

Supplementation of the culture medium with only 2% of fetal bovine serum without additional endothelial growth factors minimized proliferative signaling of the culture medium. Since changing the media of cultured HUVEC to serum-depleted milieu rapidly increased the number of detached cells, we could not extend a period of starvation nor further lower the FBS concentration (< 1%), as done in some studies (27). The rapidity of apoptosis was markedly decreased when cells were plated on 2%-gelatin-coated dishes in some of our later experiments. Because we did not use serum-concentration under 2%, the partial activity of controls (*Fig.2A, upper panel, lane 9*) could have been due to the presence of growth factors in conditioning medium, which contained 2% of fetal bovine serum (FBS).

10.1.6. Cell stimulations

Time dependence

The stimulating effect of 17-β-estradiol on phosphorylation of erk-2 kinase in COS-7 cells was apparent after 2 minutes of cell conditioning (14). The kinase activity in MCF-7 cells was greatly reduced after 60 minutes of 17-β-estradiol treatment (22). The timing for our study was chosen in order to detect the earliest 17-β-estradiol-activation in endothelial cells as well as its eventual declined or sustained effect after 60 minutes of stimulation.

Also, a time-dependent assay in HUVEC was performed (*Fig. 2C*). The results support our findings in BAMEC, where activity appeared as fast as 1 minute after 17-β-estradiol stimulation, culminated at 30 minutes and declined slightly at 60 minutes.

Dose-response

The concentration-dependence of 17-β-estradiol stimulation was conducted in HUVEC. To avoid the use of organic solvent, 17-β-estradiol was added to the incubation medium encapsulated within 2-hydroxypropyl-β-cyclodextrin 'carrier' molecule, which is water-soluble. Parallel control plates were incubated with 2-hydroxypropyl-β-cyclodextrin alone. The 17-β-estradiol concentrations chosen for activation ranged from 10^{-10} – 10^{-7} M which *in vivo* covers physiological to slightly supraphysiological doses in order to see if increasing concentrations of 17-β-estradiol contributes further to its agonist effect. The antagonists 1 μM of Wortmannin, 0.01 nM of ICI 182,780 and in later experiments 1 μM of Tamoxifen, were used to assess involvement of PI(3)K and estrogen receptors, respectively, in 17-β-estradiol-consequent phosphorylation of AKT, eNOS and in increase of NO.

10.1.7. Inhibitors

Wortmannin was isolated as an antifungal antibiotic *Penicillium wortmannii*. The specificity of Wortmannin was confirmed in a study comparing its effects on PI(3)K with other protein kinases. 1 - 10 nM of Wortmannin suppressed calf thymus PI(3)K, but had no effects on cyclic AMP-dependent protein kinase, cyclic GMP-dependent protein kinase and Ca^{2+}/calmodulin-dependent protein kinase. Thus, Wortmannin is a potent and selective inhibitor of PI(3)K. The inhibition of PI(3)K by Wortmannin is irreversible (101). Moreover, Wortmannin does not have a general cytotoxic action on the cells (107). Wortmannin, a pharmacological inhibitor of PI(3)K, was used in cell cultures to determine the involvement of active AKT after stimulation with shear stress (25;70;107), growth factors (23;25-27;70;79;79) or estradiol (26;27). Its efficiency to prevent AKT/PKB pathway activation was also confirmed in a bioassay system with endothelium-intact pig epicardial artery segments, where Wortmannin abolished the shear-stress induced production of NO (25).

We used the antagonist Wortmannin to specify that NO is released from endothelial cells upon 17-β-estradiol action through AKT/PKB pathway. In the first experiment,

cells were conditioned with 50 μM (23) or 20 μM (25) of Wortmannin, but this caused morphological deformations of cells, which we confirmed when cells were routinely checked under a microscope. In further experiments we used concentrations of Wortmannin lowered gradually to 1- 0.1 μM. The inhibitory capacity of Wortmannin remained still very efficient, but without toxic effects on stimulating cells. In some published studies Wortmannin inhibited the PI(3)K and eNOS activation in HUVEC induced by shear stress at concentrations as low as 20 nM (79). Through all experiments, Wortmannin suppressed 17-β-estradiol induced phosphorylation of both, AKT and NOS, as well as NO generation, even below unstimulated controls. This accounts for a fact, that Wortmannin additionally prevents serum-stimulated AKT autophosphorylation mediated by PI(3)K (107).

The estrogen receptor inhibitor ICI 182,780 given to us by Dr. Giguère, was already prepared in 1 μM of stock solution (solvent was ethanol). Given the volume of medium used for conditioning the cells (5 ml), the final concentration of ICI 182,780 would have less than 1 nM, which could partially explain our observation that AKT was only weakly inhibited by 17-β-estradiol stimulation (*Fig.2A*). In fact, we used a lower concentration of inhibitor then the others. For example, 1 μM of ICI 182,780 was used to block AKT phosphorylation by 17-β-estradiol in TRLEC (26), or 10 μM of ICI 182,780 was used to inhibit enzymatic activity of AKT in EA.hy.926 monolayers (91). The ability of ICI 182,780 to exhibit inhibitory effect after 17-β-estradiol stimulation also could be influenced by selection of cell types, not only by the concentrations used. Although ICI 182,780, is generally an acknowledged specific estrogen receptor inhibitor (108), in the literature there are similar observations to ours, that ICI 182,780 blocked incompletely several times. For example, 17-β-estradiol stimulated adenylate cyclase activity was not inhibited with 0.01 μM of ICI 182,780 in membranes prepared from CHO cells (20). The formation of NO upon 17-β-estradiol stimulation (of intracellular Ca^{2+} flux), via membrane ER in human monocytes, was not blocked with the inhibitor ICI 182,780 in doses 1 or 10 nM (109), and reviewed in (110). Activation of the MAP kinase pathway, which is not inhibited by the classic ER antagonist ICI 182,780, could have been responsible for its failure to inhibit 17-β-estradiol stimulation (16), and reviewed in (110). Lastly, because 17-

β-estradiol stimulated AKT phosphorylation in cortical neurons was not inhibited with 1 μM of ICI 182,780, some investigators suggest that activation of PI(3)K and AKT may not be mediated through the ER (111).

10.1.8. Western immunoblotting

To assess functioning of the AKT/PKB cascade on release of NO by the 17-β-estradiol activation via phosphorylation of AKT and subsequently phosphorylation of eNOS, we chose two specific antibodies directed against residues shown to be responsible for the activation of the phosphorylation process on both, AKT or eNOS. Because these studies explored the mechanism of the same pathway, as in our study, which is AKT/PKB signaling on eNOS phosphorylation and NO increased production in response to proliferative stimuli, we considered the use of both antibodies to be adequate and highly specific for determining that the stimulation effects of 17-β-estradiol act through AKT and eNOS in the AKT/PKB signaling pathway. The following paragraphs give a short overview about assessing the specificity of both antibodies in the current literature.

AKT phosphorylation

To determine AKT phosphorylation by 17-β-estradiol stimulation in western analysis, we used antibodies, directed against phospho-AKT (Ser 473). The reason why we used the Ab against epitope Ser 473 is, that one downstream effector of PI(3)K is the serine/threonine kinase AKT. Upon stimulation of PI(3)K, AKT becomes phosphorylated at two residues, Thr^{308} and Ser^{473} (71). In Cos-7 cells transfected with wild type AKT (HA-AKT) or kinase inactive AKT (HA-AKT K179M), the activity of AKT was determined by western blotting with a phospho-AKT-specific antibody, which recognizes serine 473 and by AKT *in vitro* kinase activity assays (24). Mutagenesis of serine 473 and threonine 308 amino acids to alanine revealed that both are required for full AKT activation. The site on serine 473 is autonomously

regulated and is not an autophosphorylation site dependent on threonine 308 phosphorylation (67).

eNOS phosphorylation

We assessed the phosphorylation of eNOS in HUVEC by western immunoblotting with specific anti-phospho-eNOS (Ser 1177) antibody.
Recently it has been shown that different proliferative stimuli through AKT/PKB pathway can phosphorylate endothelial nitric oxide synthase (eNOS) on serine 1177 (serine 1177 in human or serine 1179 in the bovine ortholog, respectively) (25). Mutation of Ser 1177 in construct where serine 1177 is replaced with alanine, decreased AKT-dependent phosphorylation of eNOS in human endothelial cells (25). Mutation of serine 1179 to alanine in co-transfected COS cells abolished AKT-dependent phosphorylation of eNOS (24).

Quantification of western immunoblotting

We realize, that western immunoblot analysis is a semi-quantitative method. For this reason, all phospho-blots were stripped and reprobed for total AKT or eNOS. This procedure allowed us to insure, that expressed ratio between phosphorylated and total protein reflects the same band. Probing for total protein showed equality of protein loading.

10.1.9. NOS activity measurement

NOS activity was measured in lysates of BAMEC in whole-cell homogenates(~100 μg total protein) by citrullin conversion assay (30;87), as described in Methods chapter in detail. The same assay in HUVEC gave inconsistent results. A likely

explanation for our inability to detect NOS activity reliable in HUVEC is that the maximal amount of total protein used from these cells was only ~50 μg. For measurement of NOS activity in cell homogenates of HUVEC usually require at least ~200 - 250 μg total protein (30;112), and in some studies ~500 – 1000 μg total protein (36). The NOS activity measurement in BAMEC needs to be repeated for statistical evaluation of 17-β-estradiol effect on NOS activity increase.

10.2. Comparison with literature

We explored the new possible mechanism through AKT/PKB pathway between NO production and 17-β-estradiol effects on vasculature. Recently, three other groups have shown that AKT/PKB can mediate rapid, non-genomic activation of eNOS by estrogen (26;27;91).

Simoncini et al. showed in 17-β-estradiol-stimulated HUVEC that eNOS activity is increased in the concentration-dependent manner, with maximum activity within 10^{-8}-10^{-7} M of 17-β-estradiol concentration, which corresponds to ≈5.5 fold of control. The NOS activity increased 2-fold in the first two minutes and peaked at 20 minutes and declined to 30 minutes of 17-β-estradiol-stimulation. Wortmannin efficiently blocked PI(3)K, but not earlier than 15 minutes after 17-β-estradiol stimulation. Its inhibitory effect in the first 15 minutes of 17-β-estradiol stimulation was only weak. For this reason, they suggest that the initial increase of eNOS activity is mediated by MAP kinases. Only the second increase in eNOS activity would be due to PI(3) activation. The ER inhibitor ICI 182,780 blocked eNOS activity.

We saw in BAMEC ≈1.5-fold increase in eNOS activity as early as 1 minute of 17-β-estradiol stimulation, this effect was maintained through 30 minutes. The maximum eNOS activity was increased ≈2.6-fold after 60 minutes of stimulation with 10^{-8} M of 17-β-estradiol. Wortmannin blocked this effect. However, we did not perform time-effect of inhibitory effects of Wortmannin on PI(3)K to determine whether other

pathways, for example MAPK, are involved in the early stages of 17-β-estradiol stimulation.

To determine the role of ER in the increase of eNOS after 17-β-estradiol stimulation through p85α, which is the subunit of PI(3)K, Simoncini transfected murine fibroblasts with ER-α and eNOS complementary DNAs. 17-β-estradiol produced 8-fold increase in eNOS activity in wild type but not in p85α-deficient fibroblasts. Furthermore, in p85α-deficient fibroblasts, co-transfection of p85α cDNA led to a 4-fold increase in 17-β-estradiol stimulated eNOS activity. The eNOS activity was suppressed in wild type and co-transfected murine fibroblasts that lacked ER-α.

In non-transfected human EC, 17-β-estradiol increased activity of PtdIns(3,4,5)P_3, that mediate the PI(3)K activation. The increase was time-dependent with maximum activity at 20 minutes, which correlated to the previously seen increase in eNOS activity in Wortmannin inhibited phase; thus this process was PI(3)K dependent. By immunoprecipitation it was shown that increases in PtdIns(3,4,5)P_3 correlate in time-dependent manner with the ligand dependent increases in ER-α associated PI(3)K activity. This process was inhibited by ER inhibitors, ICI 182,780 and Tamoxifen. Both, MAP-kinase inhibitor PD 98059 and transcriptional inhibitor actinomycin D, did not inhibite expression of PtdIns(3,4,5)*P3*, which suggests that PI(3)K is recruited by ER.

In non-transfected HUVEC 17-β-estradiol stimulated AKT-kinase activity in a time-delayed manner, the AKT activity peaked at 20 minutes after stimulation, which corresponded with peak observed in eNOS activity and PtdIns(3,4,5)P_3. To determine AKT as a mediator of 17-β-estradiol stimulation, BAEC were transiently transfected with adenoviruses containing constitutively active (myr) and dominant-negative (dn) AKT mutants. Transfection of these cells with myr-AKT produced ≈12-fold increase in eNOS activity, whereas dn-AKT mutant completely abolished 17-β-estradiol stimulated eNOS activity.

We showed by western analysis that AKT is directly activated in bovine endothelial cells as early as 1 minute after stimulation with 17-β-estradiol in time-dependent manner. The maximal level of phosphorylation of AKT ($P<0.05$, n = 4) was induced

in HUVEC by physiological concentrations of 17-β-estradiol (10^{-8} M). Further increase of 17-β-estradiol concentration (10^{-7} M) did not augment these activities, as we show by dose-response curves for AKT phosphorylation.

To assess the physiological significance of this pathway *Simoncini et al.* established in a model of ischaemia and reperfusion (I/R) injury in the mouse cremaster muscle. Treatment with 17-β-estradiol increased eNOS activity 3.2-fold and prevented the subsequent changes in leukocyte accumulation and rolling velocity after I/R. Wortmannin, the PI(3)K inhibitor, or L-NAME, the NOS inhibitor, applied to the cremaster muscle, did not cause any changes in leukocyte rolling velocity and leukocyte adherence between untreated and 17-β-estradiol treated mice after I/R. These findings indicate that the NO-induced vascular protective effect of estrogen is mediated by PI(3)K (27).

The physiological relevance of 17-β-estradiol on NO-dependent relaxation of intact blood vessels was examined in our study by Ach-mediated relaxation of rat aorta, as discussed in details earlier in this chapter. There was a 4.5-fold increase in Ach-mediated NO–dependent vasodilatation of vessels treated with 17-β-estradiol. The vasorelaxant increase was statistically significant ($P<0.05$). To determine the role of AKT/PKB kinases in modulating NO-mediated vasodilatation after the application of 17-β-estradiol, we investigated the effect of PI(3)K inhibitor. Wortmannin, when used with 17-β-estradiol for pretreatment of vessels, abrogated previously present Ach-induced vasodilatation of 17-β-estradiol treated rat aortic segments. Therefore, we demonstrated that increase in NO production and vasorelaxation after acute 17-β-estradiol exposure implicates AKT/PKB signaling pathway.

Haynes et al. showed in pretreated human endothelial cells hybridoma line EA.hy926 that 17-β-estradiol induces >4-fold NO release which was inhibitable by both, ICI 182,780 and LY294002. Therefore NO production in 17-β-estradiol-stimulated cells is mediated through both, ER and PI(3)K. To directly establish that 17-β-estradiol-triggered signal transduction pathway resulted in NO release through the AKT, EA.hy926 cells were transduced with adenoviral vectors encoding AKT kinase-inactive mutant AA-AKT which largely abrogated 17-β-estradiol-stimulated NO production. This demonstrated that functionally active AKT is required for 17-β-

estradiol-stimulated NO production. To establish the importance of membrane-associated surface receptor, cells overexpressing kinase-inactive AKT or β-gal-expressing adenovirus, were stimulated with BSA-conjugated, membrane-impermeant 17-β-estradiol. NO release increased >4-fold of control in cells transduced with β-gal upon BSA-17-β-estradiol stimulation which indicated that estradiol can stimulate an increase in NO via a surface receptor (19).

In our study, we also determined nitrite accumulation as consequence of 17-β-estradiol conditioning of HUVEC. We measured nitrite accumulation induced by 17-β-estradiol in a dose-dependent manner. When compared to the same 17-β-estradiol concentration (10 nM) as used in the above-mentioned study, we found ≈2.5-fold increase in nitrite accumulation. However, in even the lower 17-β-estradiol concentration of 1 nM increased nitrite accumulation by ≈3.5-fold ($P<0.05$, n =3). Accumulation was efficiently blocked by Wortmannin, and both, ICI 182,780 and Tamoxifen, which confirmed that the involvement of AKT/PKB pathway is estrogen receptor dependent. Considering concentration of 17-β-estradiol which was used to assess nitrite accumulation in stimulated cells, and choice of cell system, our study precedes the others, which determined NO only after stimulation with higher concentrations of 17-β-estradiol and in less physiologically relevant cell systems (19;26). In preliminary experiment, we also performed time effect (1, 30, 60 minutes) of 10 nM of 17-β-estradiol on NO generation in HUVEC. The efficient accumulation of NO was after 60 minutes of stimulation with 17-β-estradiol.

Similar to our study, *Haynes et al.* determined AKT phosphorylation in endothelial cells by stimulation with as little as 0.1 nM of 17-β-estradiol. Comparable concentration of 17-β-estradiol (10 nM) in our study induced phosphorylation of AKT >2-fold as early as 1 minute after stimulation of BAMEC if compared with findings of others when they could detect AKT phosphorylation in EC only after 5 minutes in EA.hy.926 (19) and after 15 minutes in HUVEC (26;27).

Whether 17-β-estradiol stimulation does result in eNOS phosphorylation in endothelial cells, they exposed EA.hy.926 monolayers to 10 nM of 17-β-estradiol. Phosphorylation of eNOS was detectable after 5 minutes of stimulation. We found in

HUVEC maximum eNOS phosphorylation with the same 17-β-estradiol concentration (10 nM). In our study, the increase in eNOS phosphorylation was >4-fold of control, (P<0.05, n =5). Moreover, we also showed that activation of eNOS with 17-β-estradiol was dose-dependent, for 17-β-estradiol stimulation with concentrations in physiological range. Time-dependence of eNOS phosphorylation in our preliminary experiment showed similar tendencies, with 17-β-estradiol stimulation eNOS was activated in the first 30 minutes.

The engagement of a surface ER in induction of eNOS phosphorylation by 17-β-estradiol stimulation, was shown on EA.hy.926 monolayers, which were stimulated with membrane-impermeant BSA-conjugated, 17-β-estradiol. Phosphorylation of eNOS was detectable after 5 minutes and intensified up to 30 minutes. Thus, membrane associated ER was responsible for activation of AKT cascade, eNOS phosphorylation and NO production in consequence of stimulation of endothelial cells with 17-β-estradiol.

Hisamoto et al. studied estrogen-induced AKT dependent activation of NOS in HUVEC and TRLEC. Only 17-β-estradiol, but not 17-α-estradiol caused the induction of acute activation of eNOS. In HUVEC, after stimulation with 100 nM of 17-β-estradiol, eNOS activity culminated after 15 minutes with maximum increase ≈2-fold of control, and declined slowly at 60 minutes. 17-β-estradiol induced eNOS activation in a dose-dependent manner and 10 nM of 17-β-estradiol increased eNOS activity ≈1.5-fold. If we compare with the same 17-β-estradiol concentration (10nM), we found ≈2.6-fold increase of eNOS activity in BAMEC after 60-minute of stimulation. Through all their study they used the slightly supraphysiological concentration of 17-β-estradiol of 100 nM.

The phosphorylation of AKT was found after stimulation with 100 nM of 17-β-estradiol in TRLEC only after 10 minutes, and in HUVEC, after 15 minutes. In comparison, we found a 2-fold increase in phosphorylated AKT as early as 1 minute after stimulation with 10 nM of 17-β-estradiol in BAMEC. The specificity of the augmentation of AKT activation by 17-β-estradiol was confirmed by specific inhibitory effect of Wortmannin on PI(3)K as in our study.

To assess the AKT-dependent eNOS phosphorylation, they assayed transfected TRLEC. The increased eNOS phosphorylation was found in immunoprecipitates prepared from TRLEC transfected with wild type AKT. Transfection with kinase-inactive AKT failed to phosphorylate eNOS induced by 100 nM of 17-β-estradiol. Mutation of serine 1179 to alanine in TRLEC markedly reduced 17-β-estradiol-induced phosphorylation of eNOS compared with wild type protein.

To determine the potential role of ER-α or ER-β, they transfected TRLEC with ERs. Transfection with ER-β had no effect on 17-β-estradiol-induced AKT activation. Transfection with ER-α into TRLEC caused an increase in both basal and 17-β-estradiol-induced AKT activation compared with transfection of control vector. Moreover, transfection with ER-α caused a ≈2-fold increase in eNOS activity compared with transfection of ER-β. To confirm these findings, they transfected CHO that do not express ERs, with either ER-α or ER-β. Only transfection with ER-α induced increase in AKT activity upon brief stimulation with 100 nM of 17-β-estradiol.

As already mentioned earlier in this chapter, in both cell lines used in our study, by RT-PCR we found a presence of both types of ERs, only weak ER-α (+) and predominant ER-β (+++). Because we assessed increases in NO as a consequence of activation of eNOS and AKT via PI(3)K in cell lines apparently expressing more of ER-β which is in controversy with the latest findings of some (26), more studies are required to specify, which of ERs is responsible for AKT/PKB signaling to endothelial NO release in BAMEC and HUVEC.

10.3. Significance

The objective of this study was to determine the mechanism of short-term administration of estrogen on endothelium-mediated dilatation in the ovariectomized rat aorta. Indeed, we found that endothelial and NO-dependent dilatation increased after treatment with estrogen. This direct effect of estrogen on the vasculature could

promote vasodilatation and inhibit the development and progression of arteriosclerosis. NO is an important atheroprotective molecule that causes the dilatation of blood vessels by modulating endothelial NO production.

One mechanism is the direct effect on L-type calcium channels, but it is seen only at supraphysiological concentrations of estrogens (8). At physiological concentrations, estrogen stimulates the opening of calcium-activated potassium channels through a NO-cGMP-dependent pathway, thus relaxing smooth muscle and promoting vasodilatation (39-43).

In our study, we correlated modulation of NO release with activation of AKT/PKB pathway by estrogen. Because estrogen is known to regulate NO release by eNOS (30), we show that at least estrogen partially does this by the AKT/PKB pathway. Membrane bound ER activates tyrosine kinase and could therefore activate PI(3)K (14). This effect requires ER, but the ER does not have to be transcriptionally active (113). In agreement with other studies, which show that VEGF, fluid shear stress, IGF-1 or estrogen can stimulate AKT/PKB pathway (19;24;25;70), we determined in endothelial cells that AKT phosphorylation and subsequent eNOS phosphorylation results in eNOS activation by estrogen. Moreover, we also show that production of NO is required for the rat aorta endothelial relaxation mediated by estrogen. Estrogen-induced eNOS phosphorylation and NO release were inhibited by Wortmannin, suggesting that estrogen signals via PI(3)K/AKT leads to eNOS phosphorylation and NO production. Thus, acute proangiogenic action of estrogen is mediated by AKT/PKB signaling through phosphorylation of eNOS and a subsequent increase of NO production in endothelium.

However, there are indications, that other signaling mechanism could be involved in engagement of eNOS by agonists that stimulate production of nitric oxide. The heterocomplex of heat-shock protein (Hsp90) with eNOS enhances eNOS activation by growth factors in EC. The interaction of the ER ligand-binding domain with Hsp90 has been already characterized (64). Hsp90 was found to be a part of the multimeric complex consisting of ER and B-Raf, a member of the tyrosine signaling pathway in cortical neurons (16). Hsp90, determined in EC as a heterotrimeric complex, influenced the inhibitory actions of caveolin-1 on eNOS (114). In HUVEC,

17-β-estradiol induced an Hsp90-eNOS association, mediated by ER (65). The binding of Hsp90 to eNOS increases NO production (84). Agonists, which stimulate NO, such as VEGF and shear stress, rapidly recruit Hsp90 to eNOS (84). It was shown, that NO-dependent vasorelaxation of intact rat aorta is attenuated by geldanamycin, a specific inhibitor of Hsp90 (84). Since estrogen binding to ER releases Hsp90 (63), this could contribute to the increase in NO with estrogen. Thus, Hsp90 can be considered to be another new intermediate in the mechanical signaling cascades leading to endothelial NO release induced by estrogens.

Engagement of AKT/PKB pathway in cellular signaling is now well established. The activation of AKT/PKB leads to increase proliferation of cells and less apoptosis (23). PI(3)K-stimulated AKT phosphorylation was shown to play a key role in preventing apoptosis (67;68;75). We showed in our study that estrogen activated the phosphorylation of AKT in both bovine and human EC. We also showed phosphorylation of eNOS and subsequent increase of NO production in 17-β-estradiol-stimulated HUVEC. Morover, estradiol enhanced generation of NO derived from oophorectomized rat aorta, via activated PI(3)K. The existence of functional mechanism of eNOS activation and subsequent release of nitric oxide through anti-apoptotic AKT/PKB cascade, which controls cell survival, could at least partly explain the beneficial effects of estrogen on the vasculature.

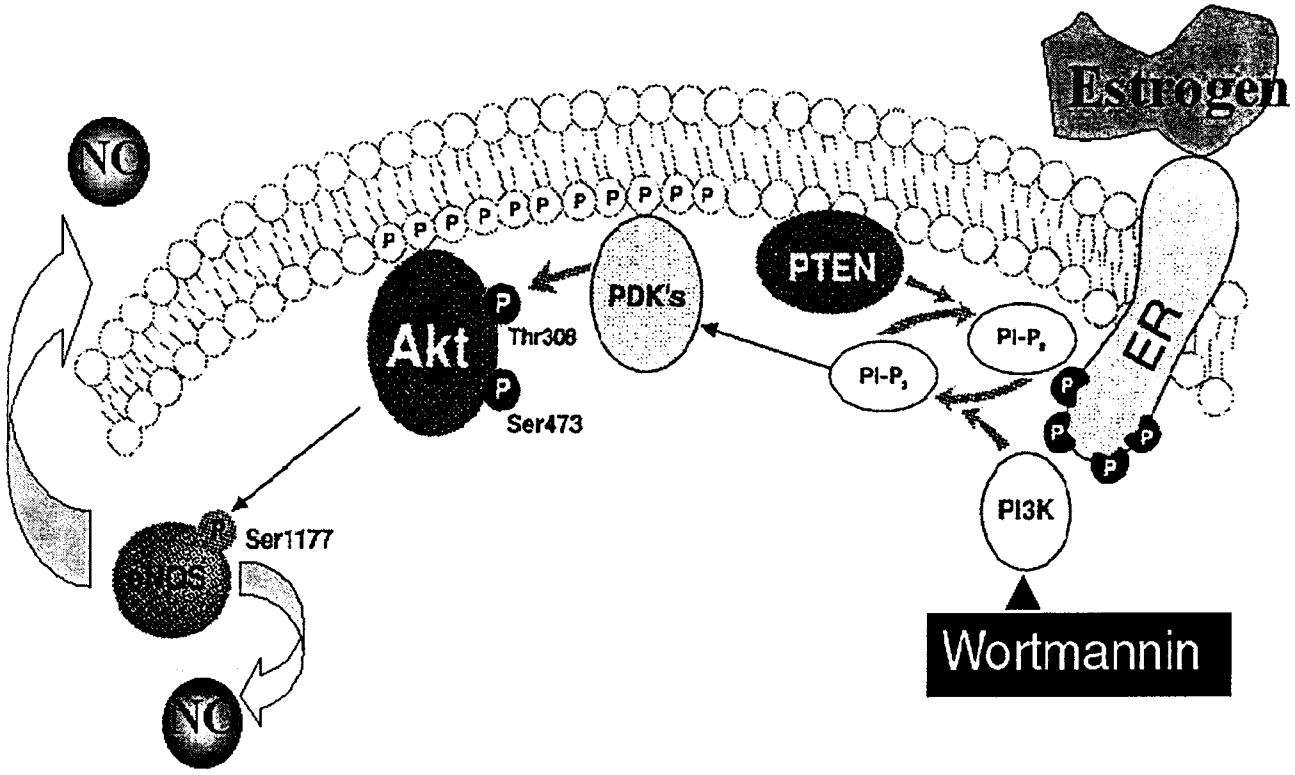
Schema Legend and Schema

Figure Legends and Figures

Schema Legend

Schema 1. The PI(3)K/AKT signaling pathway mediated by estradiol.

Steroid hormone estrogen binds to the membrane-associated estrogen receptor (ER). ER is bound in a ligand-dependent manner to the p85α regulatory subunit of (PI(3)K. Increases in PtdIns-3,4,5P_3 and 3,4P2 levels correlate with the ligand-dependent increases in ER-associated PI(3)K activity. (27) The effect of PI(3)K is completely inhibitable by PI(3)K antagonist Wortmannin. PI(3)K catalyzes the phosphorylation of the inositol ring of phosphatidylinositol lipids (P) producing PIP3 and PIP2. A downstream effector of PI(3)-kinase is the serine/threonine kinase AKT. Upon ER activation by estrogen, AKT is recruited to the plasma membrane and binds to inositol lipids via its PH domain. AKT can phosphorylate eNOS on serine 1177 in human EC or serine 1179 in bovine EC respectively, resulting in eNOS activation and NO production.(24;25) AKT phosphorylation occurs at its specific sites, Thr^{308} and Ser^{473} through an constitutively active, lipids-dependent enzyme, PDK1. This enzyme specifically phosphorylates Thr^{308}. PDK2 (not shown) is a protein kinase that targets PKB at Ser^{473}. (67) PTEN, a lipid phosphatase which dephosphorylates the PtdIns(3,4,5)P_3, acts in opposition to PI(3)K, the upstream activator of the AKT. (72)



Schema 1. **Proposed Mechanism of Activation of eNOS**

Figure 1. Time-course of AKT phosphorylation in BAMEC stimulated by 17-β-estradiol.

BAMEC treated in phenol red-free DMEM with 10 nM 17-β-estradiol for 1, 30 and 60 minutes, with 17-β-estradiol plus Wortmannin 50 μM (E/W), with Wortmannin alone (W) or with vehicle (solvents) either buffer only, ethanol, DMSO or in combination of all solvents. **A.** *upper panel* of western blot shows phospho-AKT with a weak signal of solvent treated cells (*lanes 1 - 4*). Expression of phospho-AKT in17-β-estradiol stimulated BAMEC in 1, 30 and 60 minutes (*lanes 6 - 8*). 17-β-estradiol with Wortmannin (E/W) and Wortmannin alone (W), after 30 minutes of cell stimulation, completely abrogated the induction of phosphorylation of AKT (*lanes 9 - 10*). *Lower panel*, an immunoblot with anti-AKT indicates equal total AKT protein expression. in controls (solvent) or in 17-β-estradiol (10 nM) stimulated cells. **B.** Progressive time-dependent increase in AKT phosphorylation with no activity in untreated cells (controls), increased phosphorylation during 1 minute and 30 minutes of 17-β-estradiol 10 nM stimulation. After 60 minutes of cell treatment, AKT phosphorylation maintained plateau. Arrow *(lane 5)* indicates a pre-stained marker. Data for graph are calculated from measured optical density of bands. Data represent 2 individual experiments.

Fig. 1A

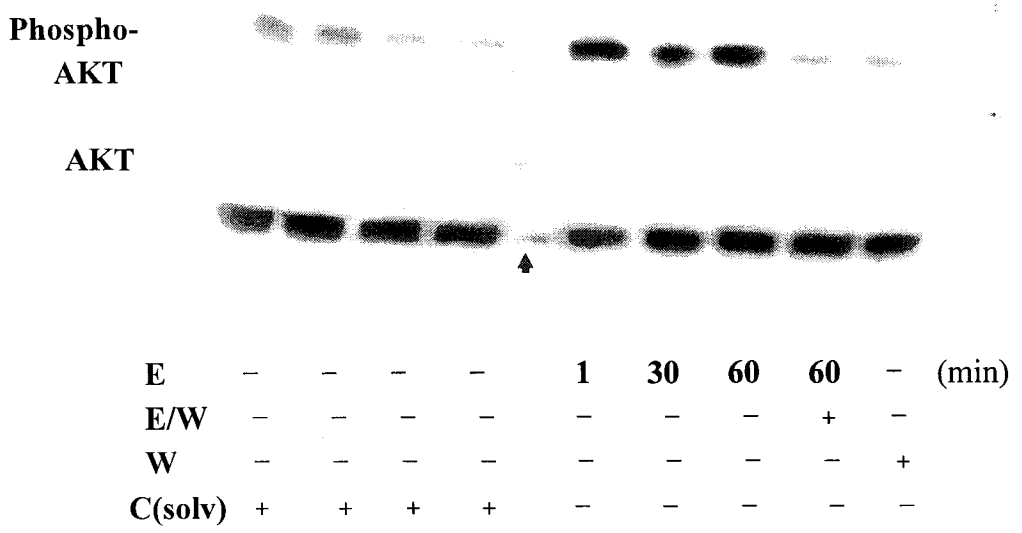


Fig. 1B

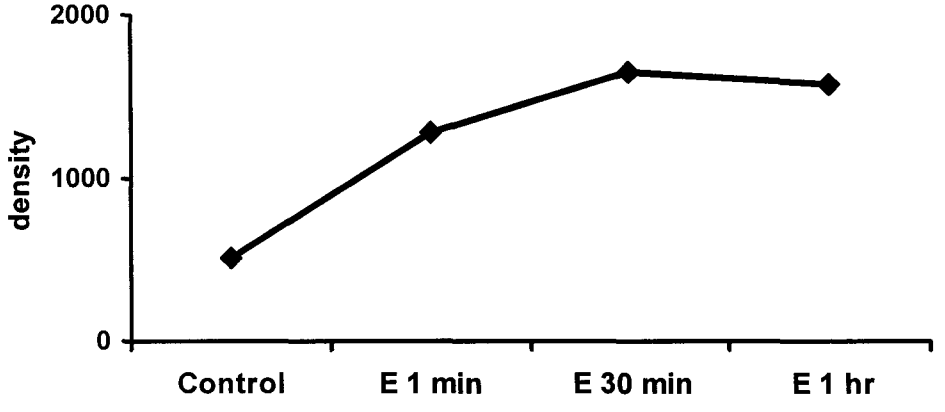
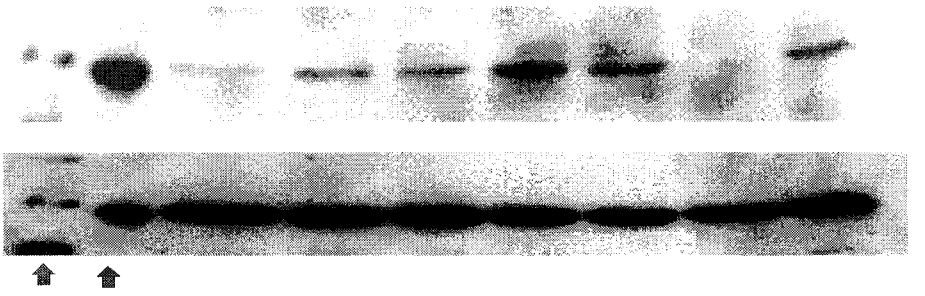


Figure 1. Time-course of AKT phosphorylation in BAMEC 17-β-estradiol stimulated.

Figure 2. 17-β-Estradiol stimulated the AKT phosphorylation in HUVEC in dose dependent manner.

HUVEC treated with different concentrations of 17-β-estradiol or inhibitors, lysates were subjected to western immunoblotting and probed with: phospho-AKT (Ser 473) **A.** *upper panel*: Arrows indicate: in the *lane 1* the marker of molecular weight, in the *lane 2* the positive control of AKT phosphorylation (NIH3T3 PDGF-treated cells). *Lanes 3 - 6*: 0.1, 1, 10, 100 nM 17-β-estradiol stimulated HUVEC for 1 hour. *lane 7*: estrogen receptor inhibitor ICI 182,780 0.01 μM with 10 nM of 17-β-estradiol 10 nM (E/ICI), *lane 8*: PI(3)K inhibitor Wortmannin 50 μM plus 17-β-estradiol 10 nM (E/W), *lane 9*: cells incubated with solvent (C) alone. *Lower panel*: immunoblots were stripped and reprobed with anti-AKT Ab. **B.** A column graph represents mean ± SEM values of optical density measured in four separate experiments. **C.** *upper panel*: HUVEC treated with 0.1, 1 (*lanes 2 – 3*) and 10 nM of 17-β-estradiol in 1, 30 and 60 minutes with 17-β-estradiol 10 nM (*lanes 4 – 6*); inhibitor Tamoxifen 1 μM with 17-β-estradiol 10 nM (*lane 7*); inhibitor Wortmannin 50 μM with estradiol (*lane 8*); vehicle control (*lane 1*). Arrow indicates a pre-stained marker. *Lower panel*: re-blotting for the total AKT. Data representative of 4 separate experiments.

Fig. 2A



E	**0.1**	**1**	**10**	**100**	**10**	**10**	– (nM)
ICI 0.01 μM	–	–	–	–	+	–	–
W 1 μM	–	–	–	–	–	+	–
C	–	–	–	–	–	–	+

Fig. 2B

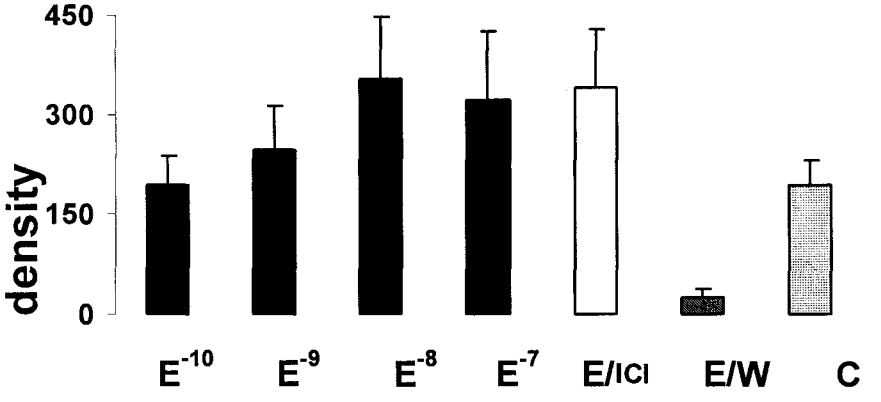
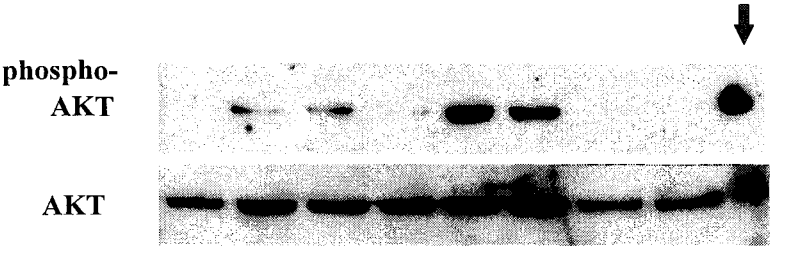


Fig. 2C



Time (min)	**60**	**60**	**60**	**1**	**30**	**60**	**30**	**30**
E (nM)	–	**0.1**	**1.0**	**10**	**10**	**10**	**10**	**10**
TMX 1 μM	–	–	–	–	–	–	+	–
W 1 μM	–	–	–	–	–	–	–	+
C	+	–	–	–	–	–	–	–

Figure 2. 17-β-Estradiol stimulated AKT phosphorylation in HUVEC.

Figure 3. eNOS phosphorylation by 17-β- estradiol is dose-dependent.

HUVEC, cultured 48 hours in phenol red-free and starved overnight in 2% of hormone-depleted FBS, were incubated with increasing concentrations of water-soluble 17-β-estradiol (cyclodextrin) for 1 hour. Simultaneously, cells in several plates were pre-incubated for 30 minutes with estrogen receptor inhibitor ICI 182,780 0.01 μM or with PI(3)K inhibitor Wortmannin 50 μM, followed by stimulation with 17-β-estradiol 10 nM. Control cells were exposed to the same conditions using solvent only. **A.** *upper panel*: cell lysates resolved on 8% SDS-PAGE and transferred to the PVDF membrane were immunoblotted with specific polyclonal anti-phospho-eNOS (Ser 1177) Ab, probed with HRP-anti-rabbit secondary Ab and visualized by enhanced chemiluminescence. *Lower panel* shows the same immunoblot, stripped, re-blocked and reprobed with monoclonal eNOS Ab to confirm equal loading of the gel. **B.** The results of densitometric analysis were expressed as ratios relative to control and presented as means ± SEM. *Significant in comparison with control ($P < 0.05$). Results are means of 5 individual experiments.

Fig. 3A

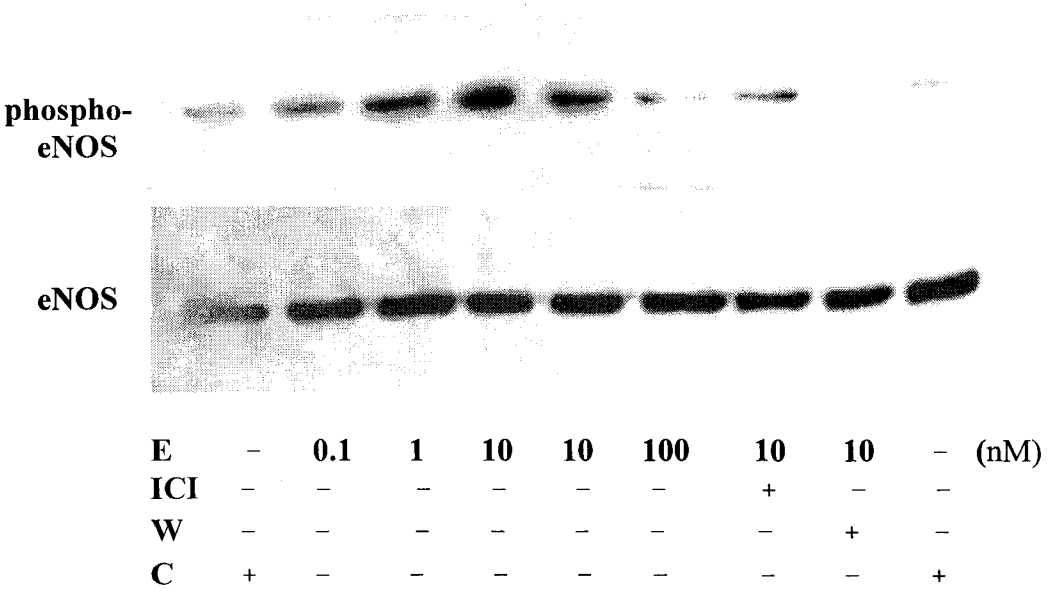


Fig. 3B

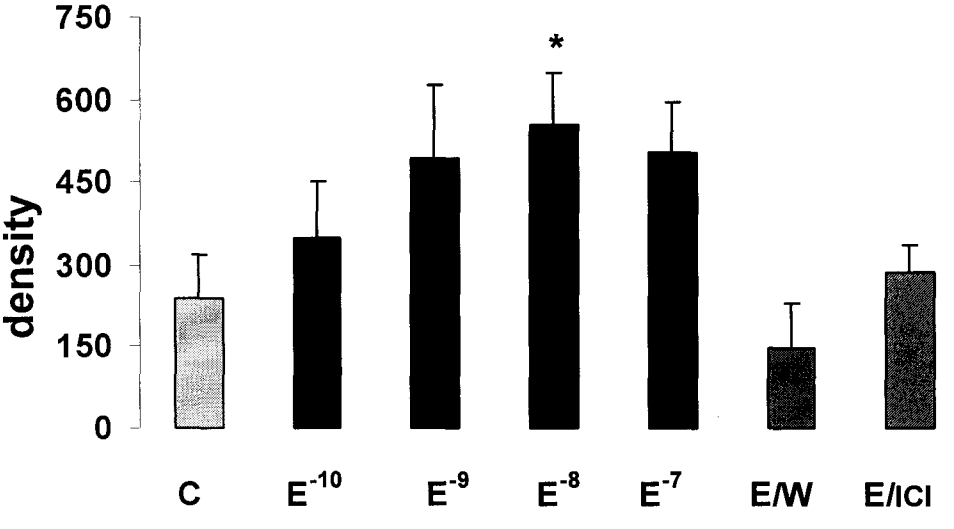


Figure 3. eNOS phosphorylation induced by 17-β-estradiol. Dose-dependence.

Figure 4. Effect of 17-β-estradiol on eNOS enzymatic activity in time.

NOS activity was measured in whole cell lysates of BAMEC incubated in 10 μM estradiol conditioned media for 1, 30 and 60 minutes. The PI(3)K/AKT inhibitor Wortmannin 50 μM + estradiol 10 μM (E/W, *hatched column*) was used in treatment of some cell plates. The NOS activity determined in cell lysates by L-[^{3}H]-arginine to L-[^{3}H]-citrulline conversion assay is expressed as fold of NOS activity measured in vehicle control after subtracting counts of background. The value of control is arbitrary set as 1 (*horizontally hatched column*). A specific NOS inhibitor L-NAME (1.5 mM) was added in parallel samples before incubation of reaction mixture. *White empty columns* show the portion of NOS activity after inhibition by L-NAME. Data represent 2 individual experiments.

Figure 5. Nitrite accumulation in the supernatant of HUVEC is estradiol-concentration dependent.

Nitrite release in the media of 17-β-estradiol increasing concentrations (10^{-10} - 10^{-7}M) treated nearly confluent HUVEC. Cell monolayers were stimulated during 1 hour with indicated doses of estradiol (expressed in nM) or in combination with inhibitors: PI(3)K/AKT inhibitor Wortmannin 50 μM (E/W), specific estrogen receptor inhibitors ICI 182,780 0.01 μM (E/ICI) or Tamoxifen 0.01 μM (E/TMX). Estradiol concentration used with all inhibitors was 10 nM. Parallel plates of solvent controls (C)(buffer only, ethanol, cyclodextrin, DMSO) were exposed to the same experimental conditions. Conditioned medias collected after cell treatment, were deproteinized, processed with Griess reagent and quantified spectrophotometrically at 540 nm as described in Methods. Background levels of solvent were subtracted from resulting NO_2^- values. Nitrite accumulation per 10^5 cells was normalized to the total volume of the incubation media. Results are mean ± SEM. *, $P<0.05$, $n = 5$.

Figure 4. Effect of 17-β-estradiol on eNOS enzymatic activity in BAMEC.

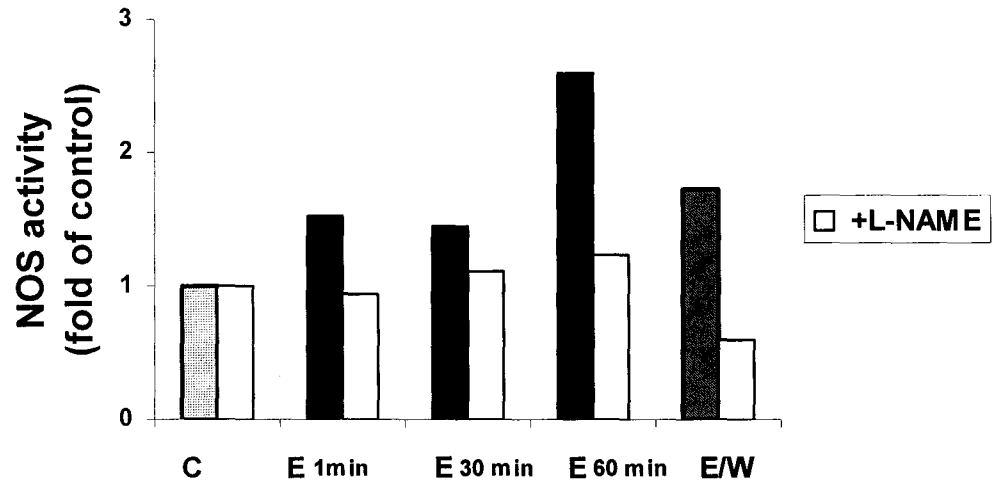


Figure 5. Nitrite accumulation induced by 17-β-estradiol in the supernatant of HUVEC.

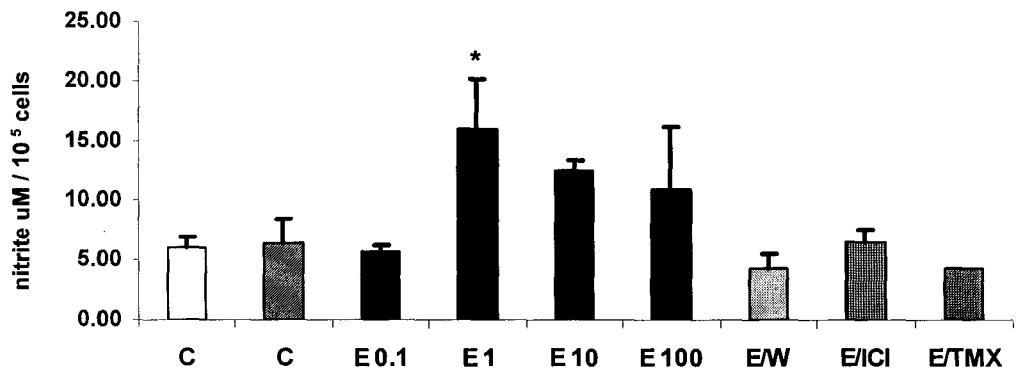


Figure 6. Bioassay trace of 17-β-estradiol induced endothelium-dependent vasorelaxation.

A. Oophorectomized rat aortic segments were equilibrated for 40 minutes at 2 gr of resting tension in a oxygenated Krebs buffer at 37°C then preconstricted with Phe 0.1 μM and a vasorelaxation dose-response curve to Ach (10^{-10} – 10^{-5} M) was determine. After washout, aortic rings were rinsed out repeatedly with fresh Krebs-bicarbonate solution and equilibrated at 2 gr of the resting tension. To the organ bath of hormone-treated aortic segments was added 10 μM of 17-β-estradiol for the 40-minute of incubation period. Control aortic segments were incubated without estradiol. Vessels were then preconstricted again with Phe 0.1 μM and Ach dose-response was repeated in both, 17-β-estradiol treated (■ *full line*) and untreated (♦*dotted line*) aortic segments. **B.** Aortic segments were preincubated for 10 minutes with Wortmannin 0.1 μM or, in other set of rings, to the Wortmannin-preincubated aortic rings was added 10 nM of 17-β-estradiol. Subsequently, Ach dose-response was created in both, Wortmannin alone incubated aortic segments (▲*broken line*) and 17-β-estradiol+Wortmannin incubated aortic segments (■ *full line*). In some experiments was used solvent (ethanol) only in the same concentration as that used for dissolving 17-β-estradiol. No dilatory effects were observed (not shown). Data represent means ± SEM. *$P<0.05$, $n = 6$.

Fig. 6A

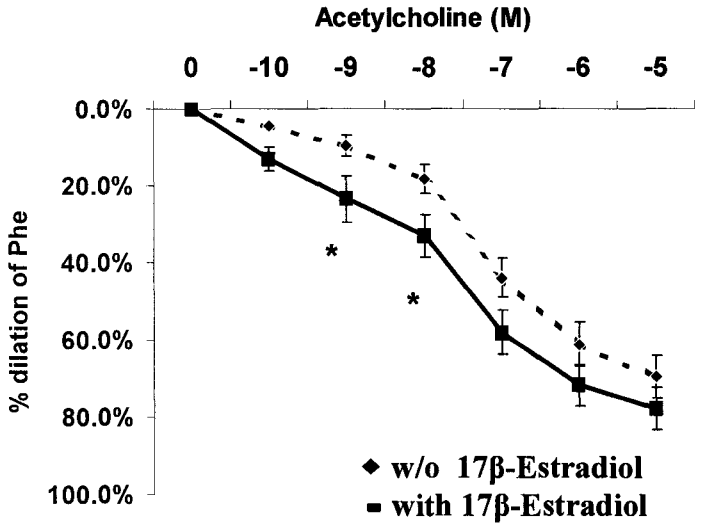


Fig. 6B

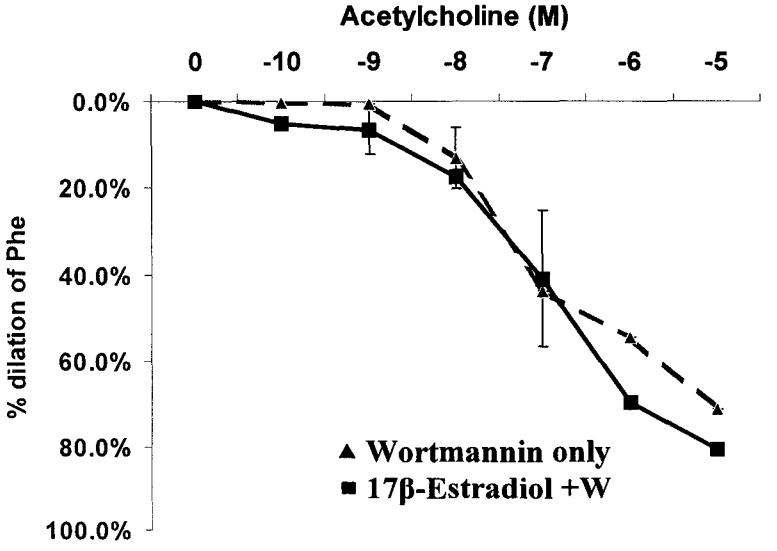


Figure 6. Bioassay on isolated rat aorta. Acute effect of 17-β-estradiol on endothelium dependent relaxation.

Appendices

Supplement to

Circulation

Volume 102 Number 18 October 31, 2000

Abstracts from Scientific Sessions 2000
Morial Convention Center
New Orleans, Louisiana
November 12–15, 2000

Named and Invited Lectures from Scientific Sessions 2000

Note: Abstract numbers are arranged to coincide with abstract titles in the *American Heart Association's Scientific Sessions 2000 Program.*

Subject and author indexes are keyed to abstract numbers. Authors of all Named and Invited Lectures and New and Young Investigator Award/Prize Abstracts are presented in the expanded Table of Contents within this Abstracts issue. Indexes are located at the back of this issue. All other supplements to this volume of *Circulation* will be indexed in the December issue.

CIRCULATION (ISSN 0009-7322) is published weekly except combined the first two weeks in January and the last two weeks in December by Lippincott Williams & Wilkins at 12107 Insurance Way, Hagerstown, MD 21740. Business offices are located at 227 East Washington Square, Philadelphia, PA 19106-3780. Production offices are located at 351 West Camden Street, Baltimore, MD 21201-2436. Individuals may subscribe for their personal use at the following rates: $177 for members of an American Heart Association scientific council and $236 for nonmembers; international: $314 for members of an American Heart Association scientific council and $418 for nonmembers. Periodicals postage paid at Hagerstown, MD, and additional mailing offices. POSTMASTER: Send address changes to CIRCULATION, American Heart Association, Lippincott Williams & Wilkins, 12107 Insurance Way, Hagerstown, MD 21740.

Endothelial Dependent Dilation by Estrogen Through the AKT/PKB Pathway.

Maria Florian, Rosario Leonor, Sheldon Magder, McGill Univ, Montreal Canada

Acute administration of estrogen is known to result in vasodilatation and release of nitric oxide but the mechanism is still unknown. We hypothesized that one mechanism might be activation of the serine-threonine kinase Akt/protien kinase B(PKB)pathway since this pathway increases ecNOS activity. To test this, we obtained aortas from oophorectomized rats which were treated with either sc. oil or 100 μg/kg of 17-β-estradiol for 8 days. The vessels were preconstricted with 10^{-7} M phenylephrine (Phe) and then we tested the vasodilator response to increasing doses of acetylcholine (Ach) (10^{-9} - 10^{-7} M) before and after a 30 minute incubation with 10^{-7} M 17-β-estradiol. This dose of estrogen by itself did not produce vasodilatation. The vasodilator response was retested after pre-treatment with Wortmannin, a PI3-K inhibitor which is the activator of AKT/PKB. Estrogen treatment resulted in a leftward shift of the acetycholine (Ach)dose response curve (EC_{50} = 0.7 X 10^{-8} M with estrogen and 0.15 x 10^{-7} M of Ach without estrogen, $P<0.05$). This effect was blocked by pre-treatment with Wortmannin. We then determined whether estrogen activates AKT/PKB in bovine microvascular endothelial cells (BMEC). Cells were treated with 2 x 10^{-8} M estradiol for 1, 30, and 60 minutes as well as in combination with 5 x 10^{-6} M Wortmannin. AKT/PKB levels were similar in all samples. With the use of a phospho-AKT/PKB antibody, we found activation of AKT/PKB as early as 1 minute with continued high activity for 60 minutes in the estradiol treated groups. This effect was blocked by Wortmannin. In conclusion the inhibition of the leftward shift of the Ach dose- response curve by Wortmannin, as well as the evidence of AKT/PKB activation in cells, suggests that this pathway may be involved in the acute affect of estrogen on nitric oxide release.

Claims to original research

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgement has been made in the text.

Reference List

1. Stampfer MJ, Colditz GA, Willett WC, Manson JE, Rosner B, Speizer FE, and Hennekens CH. Postmenopausal estrogen therapy and cardiovascular disease. Ten-year follow-up from the nurses' health study. *N Engl J Med* 325 (11):756-762, 1991.

2. Hulley S, Grady D, Bush T, Furberg C, Herrington D, Riggs B, and Vittinghoff E. Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. Heart and Estrogen/progestin Replacement Study (HERS) Research Group. *JAMA* 280 (7):605-613, 1998.

3. Herrington DM, Reboussin DM, Brosnihan KB, Sharp PC, Shumaker SA, Snyder TE, Furberg CD, Kowalchuk GJ, Stuckey TD, Rogers WJ, Givens DH, and Waters D. Effects of estrogen replacement on the progression of coronary-artery atherosclerosis. *N Engl J Med* 343 (8):522-529, 2000.

4. Alexander KP, Newby LK, Hellkamp AS, Harrington RA, Peterson ED, Kopecky S, Langer A, O'Gara P, O'Connor CM, Daly RN, Califf RM, Khan S, and Fuster V. Initiation of hormone replacement therapy after acute myocardial infarction is associated with more cardiac events during follow-up. *J Am Coll Cardiol* 38 (1):1-7, 2001.

5. Nabel EG. Coronary heart disease in women--an ounce of prevention. *N Engl J Med* 343 (8):572-574, 2000.

6. Viscoli CM, Brass LM, Kernan WN, Sarrel PM, Suissa S, and Horwitz RI. A clinical trial of estrogen-replacement therapy after ischemic stroke. *N Engl J Med* 345 (17):1243-1249, 2001.

7. Mendelsohn ME and Karas RH. Estrogen and the blood vessel wall. *Curr Opin Cardiol* 9 (5):619-626, 1994.

8. Mendelsohn ME and Karas RH. The protective effects of estrogen on the cardiovascular system. *N Engl J Med* 340 (23):1801-1811, 1999.

9. Kauser K and Rubanyi GM. Potential cellular signaling mechanisms mediating upregulation of endothelial nitric oxide production by estrogen. *J Vasc Res* 34 (3):229-236, 1997.

10. Klinge CM. Estrogen receptor interaction with estrogen response elements. *Nucleic Acids Res* 29 (14):2905-2919, 2001.

11. Robinson LJ, Weremowicz S, Morton CC, and Michel T. Isolation and chromosomal localization of the human endothelial nitric oxide synthase (NOS3) gene. *Genomics* 19 (2):350-357, 1994.

12. Lieberherr M, Grosse B, Kachkache M, and Balsan S. Cell signaling and estrogens in female rat osteoblasts: a possible involvement of unconventional nonnuclear receptors. *J Bone Miner Res* 8 (11):1365-1376, 1993.

13. Kousteni S, Bellido T, Plotkin LI, O'Brien CA, Bodenner DL, Han L, Han K, DiGregorio GB, Katzenellenbogen JA, Katzenellenbogen BS, Roberson PK, Weinstein RS, Jilka RL, and Manolagas SC. Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. *Cell* 104:719-730, 2001.

14. Migliaccio A, Di Domenico M, Castoria G, de Falco A, Bontempo P, Nola E, and Auricchio F. Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. *EMBO J* 15 (6):1292-1300, 1996.

15. Lee SJ and McEwen BS. Neurotrophic and neuroprotective actions of estrogens and their therapeutic implications. *Annu Rev Pharmacol Toxicol* 41:569-591, 2001.

16. Singh M, Setalo G Jr, Guan X, Warren M, and Toran-Allerand CD. Estrogen-induced activation of mitogen-activated protein kinase in cerebral cortical explants: convergence of estrogen and neurotrophin signaling pathways. *J Neurosci* 19 (4):1179-1188, 1999.

17. Chambliss KL, Yuhanna IS, Mineo C, Liu P, German Z, Sherman TS, Mendelsohn ME, Anderson RG, and Shaul PW. Estrogen Receptor alfa and Endothelial Nitric Oxide Synthase Are Organized Into a Functional Signaling Module in Caveolae. *Circ Res* 87 (11):e44-e52, 2000.

18. Chen Z, Yuhanna IS, Galcheva-Gargova Z, Karas RH, Mendelsohn ME, and Shaul PW. Estrogen receptor alpha mediates the nongenomic activation of endothelial nitric oxide synthase by estrogen. *J Clin Invest* 103 (3):401-406, 1999.

19. Haynes MP, Sinha D, Russell KS, Collinge M, Fulton D, Morales-Ruiz M, Sessa WC, and Bender JR. Membrane Estrogen Receptor Engagement Activates Endothelial Nitric Oxide synthase via the PI3-Kinase-Akt Pathway in human Endothelial cells. *Circ Res* 87 (8):677-682, 2000.

20. Razandi M, Pedram A, Greene GL, and Levin ER. Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: studies of ERalpha and ERbeta expressed in Chinese hamster ovary cells. *Mol Endocrinol* 13 (2Feb):307-319, 1999.

21. Auricchio F, Di Domenico M, Migliaccio A, Castoria G, and Bilancio A. The role of estradiol receptor in the proliferative activity of vanadate on MCF-7 cells. *Cell Growth Differ* 6 (2):105-113, 1995.

22. Auricchio F, Migliaccio A, Castoria G, Di Domenico M, Bilancio A, and Rotondi A. Protein tyrosine phosphorylation and estradiol action. *Ann N Y Acad Sci* 784:149-172, 1996.

23. Ahmad S, Singh N, and Glazer RI. Role of AKT1 in 17beta-estradiol- and insulin-like growth factor I (IGF-I)-dependent proliferation and prevention of apoptosis in MCF-7 breast carcinoma cells. *Biochem Pharmacol* 58 (3):425-430, 1999.

24. Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K, Franke TF, Papapetropoulos A, and Sessa WC. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* 399 (6736):597-601, 1999.

25. Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, and Zeiher AM. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* 399 (6736):601-605, 1999.

26. Hisamoto K, Ohmichi M, Kurachi H, Hayakawa J, Kanda Y, Nishio Y, Adachi K, Tasaka K, Miyoshi E, Fujiwara N, Taniguchi N, and Murata Y. Estrogen induces the akt-dependent activation of endothelial nitric-oxide synthase in vascular endothelial cells. *J Biol Chem* 276 (5):3459-3467, 2001.

27. Simoncini T, Hafezi-Moghadam A, Brazil DP, Ley K, Chin WW, and Liao JK. Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. *Nature* 407 (6803):538-541, 2000.

28. Kim HP, Lee JY, Jeong JK, Bae SW, Lee HK, and Jo I. Nongenomic stimulation of nitric oxide release by estrogen is mediated by estrogen receptor alpha localized in caveolae. *Biochem Biophys Res Commun* 263 (1):257-262, 1999.

29. M. Wehling. Specific, nongenomic actions of steroid hormones. *Annu.Rev.Physiol.* 59:365-393, 1997.

30. Caulin-Glaser T, Garcia-Cardena G, Sarrel P, Sessa WC, and Bender JR. 17 beta-estradiol regulation of human endothelial cell basal nitric oxide release, independent of cytosolic Ca2+ mobilization. *Circ Res* 81 (5):885-892, 1997.

31. J. K. Williams, M. R. Adams, D. M. Herrington, and T. B. Clarkson. Short term administration of estrogen and vascular responses of atherosclerotic coronary arteries. *J.Am.Coll.Cardiol.* 20:452-457, 1992.

32. Collins P, Rosano GM, Sarrel PM, Ulrich L, Adamopoulos S, Beale CM, McNeill JG, and Poole-Wilson PA. 17 beta-Estradiol attenuates acetylcholine-induced coronary arterial constriction in women but not men with coronary heart disease. *Circulation* 92 (1):24-30, 1995.

33. G. M. Rosano, P. M. Sarrel, P. A. Poole-Wilson, and P. Collins. Beneficial effect of oestrogen on exercise-induced myocardial ischaemia in women with coronary artery disease. *Lancet* 342:133-136, 1993.

34. J. Shan, L. M. Resnick, Q-Y. Liu, X-C. Wu, M. Barbagallo, and P. K. T. Pang. Vascular effects of 17-β estradiol in male Sprague-Dawley rats. *Am.J.Physiol.* 266:H967-H973, 1994.

35. Goetz RM, Thatte HS, Prabhakar P, Cho MR, Michel T, and Golan DE. Estradiol induces the calcium-dependent translocation of endothelial nitric oxide synthase. *Proc Natl Acad Sci U S A* 96 (6):2788-2793, 1999.

36. J. F. Arnal, S. Clamens, C. Pechet, A. Negre-Salvayre, C. Allera, J-P. Girolami, R. Salvayre, and F. Bayard. Ethinylestradiol does not enhance the expression of nitric oxide synthase in bovine endothelial cells but increases the release of bioactive nitric oxide by inhibiting superoxide anion production. *Proc.Natl.Acad.Sci.USA* 93:4108-4113, 1996.

37. Guetta V, Quyyumi AA, Prasad A, Panza JA, Waclawiw M, and Cannon RO 3rd. The role of nitric oxide in coronary vascular effects of estrogen in postmenopausal women. *Circulation* 96 (9):2795-2801, 1997.

38. L. J. Ignarro. Endothelium-derived nitric oxide: actions and properties. *FASEB J.* 3:31, 1989. (Abstract)

39. D. J. Darkow, L. Lu, and R. E. White. Estrogen relaxation of coronary artery smooth muscle is mediated by nitric oxide and cGMP. *Am.J.Physiol.* 41:H2765-H2773, 1997.

40. Rosenfeld CR, White RE, Roy T, and Cox BE. Calcium-activated potassium channels and nitric oxide coregulate estrogen-induced vasodilation. *Am J Physiol Heart Circ Physiol* 279 (1):H319-H328, 2000.

41. Rusko J, Li L, and van Breemen C. 17-beta-Estradiol stimulation of endothelial K+ channels. *Biochem Biophys Res Commun* 214 (2):367-372, 1995.

42. Wellman GC, Bonev AD, Nelson MT , and Brayden JE. Gender differences in coronary artery diameter involve estrogen, nitric oxide, and Ca(2+)-dependent K+ channels. *Circ Res* 79 (5):1024-1030, 1996.

43. White RE, Darkow DJ, and Lang JL. Estrogen relaxes coronary arteries by opening BKCa channels through a cGMP-dependent mechanism. *Circ Res* 77 (5):936-942, 1995.

44. P. Feldman, O. Griffith, and D. Stuehr. The surprising life of nitric acid. *Chemical Engineering News* 71:26-38, 1993.

45. Moncada S and Higgs A. The L-arginine-nitric oxide pathway. *N Engl J Med* 329 (27):2002-2012, 1993.

46. Moncada S, Palmer RM, and Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev* 43:109-142, 1991.

47. Furchgott RF. The discovery of endothelium-dependent relaxation. *Circulation (Supplement V)* 87 (5):V-3-V-8, 1993.

48. Stoclet JC, Muller B, Andriantsitohaina R, and Kleschyov A. Overproduction of nitric oxide in pathophysiology of blood vessels. *Biochemistry (Mosc)* 63 (7):826-832, 1998.

49. R. F. Furchgott. Role of endothelium in responses of vascular smooth muscle. *Circ.Res.* 53 (5):557-573, 1983.

50. T. Hayashi, J. M. Fukuto, I. J. Ignarro, and G. Chaudhuri. Basal release of nitric oxide from aortic rings is greater in female rabbits than in male rabbits: Implications for atherosclerosis. *Proc.Natl.Acad.Sci.USA* 89:11259-11262, 1992.

51. M. S. Mohazzab-H. K. M. Wolin. Mediation of signal transduction by oxidants. In: *Oxidative Stress and the Molecular Biology of Antioxidant Defenses*,Anonymous Cold Spring Harbor Laboratory Press, 19970, p. 21-48.

52. Nathan C and Xie QW. Regulation of biosynthesis of nitric oxide. *J Biol Chem* 269 (19):13725-13728, 1994.

53. Stuehr DJ and Griffith OW. Mammalian nitric oxide synthases. *Adv Enzymol Relat Areas Mol Biol* 65:287-346, 1992.

54. Cowley SM, Hoare S, Mosselman S, and Parker MG. Estrogen receptors alpha and beta form heterodimers on DNA. *J Biol Chem* 272 (32):19858-19862, 1997.

55. S. Kim-Schulze, K. A. McGowan, S. C. Hubchak, M. C. Cid, M. B. Martin, H. K. Kleinman, G. L. Greene, and H. W. Schnaper. Expression of an estrogen receptor by human coronary artery and umbilical vein endothelial cells. *Circulation* 94:1402-1407, 1996.

56. C. D. Venkov, A. B. Rankin, and D. E. Vaughan. Identification of authentic estrogen receptor in cultured endothelial cells. A potential mechanism for steroid hormone regulation of endothelial function. *Circulation* 94:727-733, 1996.

57. G. J. M. Kuiper, E. Enmark, M. Pelto-Huikko, and S. Nilsson. Cloning of a novel estrogen receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci USA* 93:5925-5939, 1996.

58. Couse JF, Lindzey J, Grandien K, Gustafsson JA, and Korach KS. Tissue distribution and quantitative analysis of estrogen receptor-alpha (ERalpha) and estrogen receptor-beta (ERbeta) messenger ribonucleic acid in the wild-type and ERalpha-knockout mouse. *Endocrinology* 138 (11):4613-4621, 1997.

59. Makela S, Savolainen H, Aavik E, Myllarniemi M, Strauss L, Taskinen E, Gustafsson JA, and Hayry P. Differentiation between vasculoprotective and uterotrophic effects of ligands with different binding affinities to estrogen receptors alpha and beta. *Proc Natl Acad Sci U S A* 96 (12):7077-7082, 1999.

60. Shaul PW. Rapid activation of endothelial nitric oxide synthase by estrogen. *Steroids* 64 (1-2):28-34, 1999.

61. Razandi M, Pedram A, and Levin ER. Estrogen signals to the preservation of endothelial cell form and function. *J Biol Chem* 275 (49):38540-38546, 2000.

62. Garcia-Cardena G, Fan R, Stern DF, Liu J, and Sessa WC. Endothelial nitric oxide synthase is regulated by tyrosine phosphorylation and interacts with caveolin-1. *J Biol Chem* 271 (44):27237-27240, 1996.

63. Benjamin IJ and McMillan DR. Stress (heat shock) proteins: molecular chaperones in cardiovascular biology and disease. *Circ Res* 83 (2):117-132, 1998.

64. Aumais JP, Lee HS, Lin R, and White JH. Selective interaction of hsp90 with an estrogen receptor ligand-binding domain containing a point mutation. *J Biol Chem* 272 (18):12229-12235, 1997.

65. Russell KS, Haynes MP, Caulin-Glaser T, Rosneck J, Sessa WC, and Bender JR. Estrogen stimulates heat shock protein 90 binding to endothelial nitric oxide synthase in human vascular endothelial cells. Effects on calcium sensitivity and NO release. *J Biol Chem* 275 (7):5026-5030, 2000.

66. Mendelsohn ME. Nongenomic, ER-mediated activation of endothelial nitric oxide synthase: how does it work? What does it mean? *Circ Res* 87 (11):956-960, 2000.

67. Coffer PJ, Jin J, and Woodgett JR. Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation. *Biochem J* 335 (1):13, 1998.

68. Kohn AD, Barthel A, Kovacina KS, Boge A, Wallach B, Summers SA, Birnbaum MJ, Scott PH, Lawrence JC Jr, and Roth RA. Construction and characterization of a conditionally active version of the serine/threonine kinase Akt. *J Biol Chem* 273 (19):11937-11943, 1998.

69. Kureishi Y, Luo Z, Shiojima I, Bialik A, Fulton D, Lefer DJ, Sessa WC, and Walsh K. The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals. *Nat Med.* 6 (9):1004-1010, 2000.

70. Fisslthaler B, Dimmeler S, Hermann C, Busse R, and Fleming I. Phosphorylation and activation of the endothelial nitric oxide synthase by fluid shear stress. *Acta Physiol Scand* 168 (1):81-88, 2000.

71. Stokoe D, Stephens LR, Copeland T, Gaffney PR, Reese CB, Painter GF, Holmes AB, McCormick F, and Hawkins PT. Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. *Science* 277 (5325):567-570, 1997.

72. Simpson L and Parsons R. PTEN: life as a tumor suppressor. *Exp Cell Res* 264 (1):29-41, 2001.

73. Nakatani K, Thompson DA, Barthel A, Sakaue H, Liu W, Weigel RJ, and Roth RA. Up-regulation of Akt3 in estrogen receptor-deficient breast cancers and androgen-independent prostate cancer lines. *J Biol Chem* 274 (31):21528-21532, 1999.

74. Ushio-Fukai M, Alexander RW, Akers M, Yin Q, Fujio Y, Walsh K, and Griendling KK. Reactive oxygen species mediate the activation of Akt/protein kinase B by angiotensin II in vascular smooth muscle cells. *J Biol Chem* 274 (32):22699-22704, 1999.

75. Campbell RA, Bhat-Nakshatri P, Patel NM, Constantinidou D, Ali S, and Nakshatri H. Phosphatidylinositol 3-kinase/AKT-mediated activation of estrogen receptor alpha: a new model for anti-estrogen resistance. *J Biol Chem* 276 (13):9817-9824, 2001.

76. Lobenhofer EK, Huper G, Iglehart JD, and Marks JR. Inhibition of mitogen-activated protein kinase and phosphatidylinositol 3-kinase activity in MCF-7

cells prevents estrogen-induced mitogenesis. *Cell Growth Differ* 11 (2):99-110, 2000.

77. Dupont J and Le Roith D. Insulin-like growth factor 1 and oestradiol promote cell proliferation of MCF-7 breast cancer cells: new insights into their synergistic effects. *Mol Pathol* 54 (3):149-154, 2001.

78. Hayashi T, Yamada K, Esaki T, Kuzuya M, Satake S, Ishikawa T, Hidaka H, and Iguchi A. Estrogen increases endothelial nitric oxide by a receptor-mediated system. *Biochem Biophys Res Commun* 214 (3):847-855, 1995.

79. Dimmeler S, Dernbach E, and Zeiher AM. Phosphorylation of the endothelial nitric oxide synthase at ser-1177 is required for VEGF-induced endothelial cell migration. *FEBS Lett* 477 (3):258-262, 2000.

80. Ranta V, Orpana A, Mikkola T, Ylikorkala O, and Viinikka L. Culturing of human vascular endothelial cells strongly affects their endothelin-1 and prostacyclin production. *Mol Cell Biochem* 177 (1-2):251-255, 1997.

81. Karas RH, Gauer EA, Bieber HE, Baur WE, and Mendelsohn ME. Growth factor activation of the estrogen receptor in vascular cells occurs via a mitogen-activated protein kinase-independent pathway. *J Clin Invest* 101 (12):2851-2861, 1998.

82. Figtree GA, Webb CM, and Collins P. Tamoxifen acutely relaxes coronary arteries by an endothelium-, nitric oxide-, and estrogen receptor-dependent mechanism. *J Pharmacol Exp Ther* 295 (2):519-523, 2000.

83. Seccombe JF. *Vasoactive factors produced by the endothelium : physiology and surgical implications*, Boca Raton, FL:1994. 137 pages.

84. Garcia-Cardena G, Fan R, Shah V, Sorrentino R, Cirino G, Papapetropoulos A, and Sessa WC. Dynamic activation of endothelial nitric oxide synthase by Hsp90. *Nature* 392:821-824, 1998.

85. D. Y. Cheng and C. A. Gruetter. Chronic estrogen alters contractile responsiveness to angiotensin II and norepinephrine in female rat aorta. *European Journal of Pharmacology* 215:171-176, 1992.

86. Stewart KG, Zhang Y, and Davidge ST. Estrogen decreases prostaglandin H synthase products from endothelial cells. *J Soc Gynecol Investig* 6 (6):322-327, 1999.

87. Titheradge MA. *Nitric Oxide Protocols*, 1998. 324 pages.

88. Tsukahara H, Gordienko DV, and Goligorsky M. Continuous monitoring of nitric oxide release from human umbilical vein endothelial cells. *Biochem Biophys Res Commun* 193 (2):722-729, 1993.

89. Migliaccio A, Piccolo D, Castoria G, Di Domenico M, Bilancio A, Lombardi M, Gong W, Beato M, and Auricchio F. Activation of the Src/p21^{ras}/Erk pathway by progesterone receptor via cross-talk with estrogen receptor . *EMBO J* 17 (7):2008-2018, 1998.

90. Shaul PW. Novel role of estrogen receptors in vascular endothelium. *Semin Perinatol* 24 (1):70-74, 2000.

91. Haynes MP, Russell KS, and Bender JR. Molecular mechanisms of estrogen actions on the vasculature. *J Nucl Cardiol* 7 (5):500-508, 2000.

92. Li Z and Duckles SP. Influence of gender on vascular reactivity in the rat. *J Pharmacol Exp Ther* 268:1426-1431, 1994.

93. Stallone JN, Crofton JT, and Share L. Sexual dimorphism in vasopressin-induced contraction of rat aorta. *Am J Physiol* 260 (2 Pt 2):H453-H458, 1991.

94. Huang A, Sun D, Koller A, and Kaley G. Gender difference in flow-induced dilation and regulation of shear stress: role of estrogen and nitric oxide. *Am J Physiol* 275 (5 Pt 2):R1571-R1577, 1998.

95. Tejera N, Balfagon G, Marin J, and Ferrer M. Gender differences in the endothelial regulation of alpha2-adrenoceptor-mediated contraction in the rat aorta. *Clin Sci (Colch)* 97 (1):19-25, 1999.

96. Huang A, Sun D, Koller A, and Kaley G. Gender difference in myogenic tone of rat arterioles is due to estrogen-induced, enhanced release of NO. *Am J Physiol* 272 (4 Pt 2):H1804-H1809, 1997.

97. Davidge ST, Zhang Y, and Stewart KG. A comparison of ovariectomy models for estrogen studies. *Am J Physiol Regul Integr Comp Physiol* 280 (3):R904-R907, 2001.

98. M. C. Pareses-Carbajal, M. A. Juarez-Oropeza, C. M. Ortiz-Mendoza, and D. Mascher. Effects of acute and chronic estrogenic treatment on vasomotor responses of aortic rings from ovariectomized rats. *Life Sci* 57 (5):473-486, 1995.

99. le Tran Y, Fung A, and Forster C. Role of gender and vascular endothelium in rat aorta response to 17 beta-estradiol. *Can J Physiol Pharmacol* 75 (12):1393-1397, 1997.

100. Crews JK and Khalil RA. Gender-specific inhibition of Ca2+ entry mechanisms of arterial vasoconstriction by sex hormones. *Clin Exp Pharmacol Physiol* 26 (9):707-715, 1999.

101. Yano H, Nakanishi S, Kimura K, Hanai N, Saitoh Y, Fukui Y, Nonomura Y, and Matsuda Y. Inhibition of histamine secretion by wortmannin through the blockade of phosphatidylinositol 3-kinase in RBL-2H3 cells. *J Biol Chem* 268 (34):25846-25856, 1993.

102. Orpana A, Ranta V, Mikkola T, Viinikka L, and Ylikorkala O. Inducible nitric oxide and prostacyclin productions are differently controlled by extracellular matrix and cell density in human vascular endothelial cells. *J Cell Biochem* 64 (4):538-546, 1997.

103. Fleming I, Bauersachs J, Fisslthaler B, and Busse R. Ca2+-independent activation of the endothelial nitric oxide synthase in response to tyrosine phosphatase inhibitors and fluid shear stress. *Circ Res* 82 (6):686-695, 1998.

104. Dimmeler S, Haendeler J, Nehls M, and Zeiher AM. Suppression of apoptosis by nitric oxide via inhibition of interleukin-1beta-converting enzyme (ICE)-like and cysteine protease protein (CPP)-32-like proteases. *J Exp Med* 185 (4):601-607, 1997.

105. Berthois Y, Katzenellenbogen JA, and Katzenellenbogen BS. Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. *Proc Natl Acad Sci U S A* 83 (8):2496-2500, 1986.

106. Mikkola T, Turunen P, Avela K, Orpana A, Viinikka L, and Ylikorkala O. 17 beta-estradiol stimulates prostacyclin, but not endothelin-1, production in human vascular endothelial cells. *J Clin Endocrinol Metab* 80 (6):1832-1836, 1995.

107. Dimmeler S, Assmus B, Hermann C, Haendeler J, and Zeiher AM. Fluid shear stress stimulates phosphorylation of Akt in human endothelial cells: involvement in suppression of apoptosis. *Circ Res* 83 (3):334-341, 1998.

108. Tremblay A, Tremblay GB, Labrie C, Labrie F, and Giguere V. EM-800, a novel antiestrogen, acts as a pure antagonist of the transcriptional functions of estrogen receptors alpha and beta. *Endocrinology* 139 (1):111-118, 1998.

109. Stefano GB, Prevot V, Beauvillain JC, Fimiani C, Welters I, Cadet P, Breton C, Pestel J, Salzet M, and Bilfinger TV. Estradiol coupling to human monocyte nitric oxide release is dependent on intracellular calcium transients: evidence for an estrogen surface receptor. *J Immunol* 163 (7):3758-3763, 1999.

110. Kelly MJ and Levin ER. Rapid actions of plasma membrane estrogen receptors. *Trends Endocrinol Metab* 12 (4):152-156, 2001.

111. Honda K, Sawada H, Kihara T, Urushitani M, Nakamizo T, Akaike A, and Shimohama S. Phosphatidylinositol 3-kinase mediates neuroprotection by estrogen in cultured cortical neurons. *J Neurosci Res* 60 (3):321-327, 2000.

112. Ramasamy S, Drummond GR, Ahn J, Storek M, Pohl J, Parthasarathy S, and Harrison DG. Modulation of expression of endothelial nitric oxide synthase by nordihydroguaiaretic acid, a phenolic antioxidant in cultured endothelial cells. *Mol Pharmacol* 56 (1):116-123, 1999.

113. Gray GA, Sharif I, Webb DJ, and Seckl JR. Oestrogen and the cardiovascular system: the good, the bad and the puzzling. *Trends Pharmacol Sci* 22 (3):152-155, 2001.

114. Gratton JP, Fontana J, O'Connor DS, Garcia-Cardena G, McCabe TJ, and Sessa WC. Reconstitution of an endothelial nitric-oxide synthase (eNOS), hsp90, and caveolin-1 complex in vitro. Evidence that hsp90 facilitates calmodulin stimulated displacement of eNOS from caveolin-1. *J Biol Chem* 275 (29):22268-22272, 2000.