THE ROLE OF ESTROGEN IN THE MAINTENANCE OF HEALTHY ENDOTHELIUM

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SUMMARY

The place of estrogen in women's health remains controversial. Premenopausal women have a lower prevalence of cardiovascular disease (CVD) than men and in observational studies hormone replacement therapy (HRT) decreases CVD in postmenopausal women. However, prospective randomized trials of secondary and primary prevention have failed to substantiate an overall protective effect from HRT and have even shown some harm. To explain this paradox it is necessary to better understand the effects of estrogen on the vascular wall. Estrogen rapidly mediates the activation of eNOS and increases the production of nitric oxide (NO), an important factor for endothelial health. In ovariectomized rats estrogen reduces production of superoxide (O_2^-) by NAD(P)H oxidase. The decreased function is associated with a decrease in the $p47^{phox}$ component of NAD(P)H oxidase and its interaction with the multicomponent enzyme. In these rats estrogen did not alter eNOS expression and bioavailability of NO, which is in contrast to its acute effects. This highlights the difference between chronic and acute studies. The decrease in O_2^- production suggests the intracellular signaling.

Estrogen has antiapoptotic effects. Oxidized low-density lipoprotein (oxLDL) and the inflammatory cytokine TNFα increased apoptosis which is associated with atherosclerosis. In human umbilical vein endothelial cells (HUVEC), estrogen decreased the extent of TNFα and oxLDL induced apoptosis as indicated by the expression of cleaved caspase-3 and FACS assay. Estrogen also preserves the antiapoptotic mitochondrial Bcl-2 and Bcl-xL proteins.

Estrogen has angiogenic properties that can help a healthy endothelium respond to injury. However, estrogen increases the angiogenesis caused by TNFα and this could lead to revascularization in the plaques of women with advanced disease.

Overall the balance between the positive and negative aspects of the effects of estrogen on the vascular wall could explain the paradoxical response in older women.

RÉSUMÉ

Le rôle de l'estrogène pour la santé des femmes suscite encore des débats. Les maladies cardiovasculaires (MCV) sont moins fréquentes chez les femmes pré-ménopausées que chez les hommes et des études d'observation ont constaté que la thérapie de remplacement d'hormone (TRH) réduit les MCV chez les femmes post-ménopausées. Cependant, des essais ouverts aléatoires sur les préventions secondaire et primaire n'ont pas permis d'établir un effet préventif général pour les MCV et ont même démontré de la nocivité. Pour expliquer ce paradoxe, il est nécessaire de mieux comprendre les effets de l'estrogène sur la paroi vasculaire. L'estrogène est impliqué dans l'activation rapide du monoxyde d'azote synthase (NOS) endothéliale et augmente la production du monoxyde d'azote (NO), qui est un facteur important pour la santé endothéliale. Chez les rats femelles ovariectomisées, l'estrogène réduit la production de l'anion superoxyde (O_2^-) par la NAD(P)H oxydase. Cette diminution de fonction est associée à une réduction du composant $p47^{phox}$ de la NAD(P)H oxydase, ainsi qu'à son interaction avec cette enzyme multicomposante. Chez ces rats, l'estrogène n'altère ni l'expression du NOS ni la biodisponibilité du NO, ce qui est contraire à ses effets aigus. Ces démonstrations soulignent la différence entre les études aux effets chroniques et aux effets aigus. La diminution de la production du O_2^- suggère un signalement intracellulaire.

L'estrogène a des effets antiapoptotiques. Les lipoprotéines de faible densité oxydées (oxLDL) et la cytokine inflammatoire TNFα ont augmenté l'apoptose, ce qui est associé avec l'athérosclérose. Chez les cellules endothéliales issues de veine ombilical humaine (HUVEC), l'estrogène a réduit le niveau de l'apoptose causée par TNFα et oxLDL,

comme il a été démontré par l'expression de la caspase-3 activée et par l'essai de cytométrie de flux. L'estrogène préserve également les protéines mitochondriales antiapoptotiques Bcl-2 et Bcl-xL.

L'estrogéne a des propriétés d'angiogenèse qui peuvent aider un tissu endothélial sain à réagir à une blessure. Par contre, l'estrogène augmente l'angiogenèse causée par TNFα et ceci pourrait conduire à une nouvelle vascularisation dans les plaques chez les femmes qui sont dans un état avancé de la maladie.

En général, l'équilibre entre les aspects positifs et négatifs des effets de l'estrogène sur la paroi vasculaire pourrait expliquer la réaction paradoxale chez les femmes plus âgées.

DEDICATION

This thesis is dedicated to my dearest and faithful daughter Livia, who was always enthusiastic about my work.

It is also dedicated to my patient husband Ladislav, who understood and approved the necessity to reorient my career.

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PREFACE

Contributions of Authors (alphabetically)

Livia Florianova, a co-author of my paper in chapter IV, an undergraduate student from the Department of Anatomy and Cell Biology, McGill University, she was responsible for the following experiments on the cell culture (HUVEC): wound injury / healing assay for which she acquired captions and analyzed data on the microscope; in proliferation BrdU assay she performed readings on the spectrophotometer; she determined the count of viable cells after enzymatic dissociation and analyzed data.

Anatoli Freiman, a co-author of my paper in chapter II, an undergraduate student from the Department of Physiology, McGill University, he worked on preliminary western blot experiments to determine eNOS protein expression in non-cardiovascular tissues of ovariectomized rats.

Dr Sabah Hussain, a co-author of my paper in chapter IV, a professor at McGill, he has been very productive especially in the area of angiogenesis. His last-author publications on this area appeared in excellent journals such as FASEB J. His expertise in angiogenesis was indispensable for my study in this subject matter.

Since the beginning of my graduate studies in the Physiology Department of McGill University, within the Critical Care Division of the Royal Victoria Hospital, I have been responsible or have contributed to the following publications and presentations:

Publications, Presentations, Scientific Communications

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ABBREVIATIONS

Ach – acetylcholine

AIF - apoptosis-inducing factor

AII – angiotensin-II

AKT/PKB – serine/threonine kinase, AKT/protein kinase B

Ang-1 - Angiopoietin-1

Ang-2 - Angiopoietin-2

Ang-3/4 - Angiopoietin-3/4

Angs - Angiopoietins

ANT - adenine nucleotide translocator

Apaf-1 - apoptosis protease-activating factor-1

AT_1R - AII type 1 receptor

BAEC(s) - bovine aortic endothelial cell(s)

BAMEC(s) - bovine adrenal medulla microvascular endothelial cell(s)

BAPTA-AM - acetoxymethyl esther; an intracellular Ca^{2+} chelator

bFGF – basic fibroblast growth factor

BH4 – tetrahydrobiopterin

BrdU - 5-bromo-2'-deoxyuridine

Ca^{2+} - calcium ion

CAD – coronary artery disease

CaM – calmodulin

CAM - camptothecin

cAMP - cyclic adenosine monophosphate

CGD - chronic granulomatous disease

cGMP - cyclic guanylate monophosphate

CHD - coronary heart disease

CHO - Chinese hamster ovary cells

CSS - charcoal-stripped fetal bovine serum

CSS-HI – charcoal-stripped fetal bovine serum, heat inactivated

CVD - cardiovascular disease

DERKO - double ERα/ERβ knockout

DISC – death-inducing signaling complex

DPI - diphenyleneiodonium

E2 - estrogen, 17-β-estradiol

E2-BSA - β-estradiol-17-hemisuccinate:bovine serum albumin

EA.hy 926 - permanent human endothelial hybrid cell line

EC(s) - endothelial cell(s)

ECM - extracellular matrix

EE2 – ethinylestradiol

EGTA – ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid; Ca^{2+} chelator

eNOS -endothelial nitric oxide synthase

ERβ - estrogen receptor beta

ERα,βKO - double ERα,β-knockout mice

ER(s) - estrogen receptor(s)

ERE - estrogen-response element

ERK - extracellular-regulated kinase

ERKO - estrogen receptor knockout

ERT - estrogen replacement therapy

ERα - estrogen receptor alpha

ET-1 – endothelin-1

FADD - Fas associated protein with death domain

FBF - forearm blood flow

FBS – fetal bovine serum

FGF – fibroblast growth factor

Flk-1/KDR – fetal liver kinase/kinase-insert domain receptor or VEGFR-2

Flt-1 – Fms-like tyrosine kinase-1 or VEGFR-1

Flt-4 – fms-like-tyrosine kinase receptor or VEGFR-3

FMD - flow mediated vasodilation

FOXO1 - forhead transcription factor FKHR

GC – (cytosolic) guanylate cyclase

GPCR(s) - G-protein-coupled receptor(s)

GPR - G-protein-coupled receptor

GSH - glutathione

GTN - glyceryl trinitrate

HBD – hormone-binding domaine

HDCEC - human dermal capillary endothelial cell

HDCEC(s) - human dermal capillary endothelial cell(s)

HDL - high-density lipoprotein

HERS - Heart and Estrogen-Progestin Replacement Study

HRT - hormone replacement therapy

Hsp27 - heat shock protein 27

Hsp90 - heat shock protein 90

HUVEC(s) - human umbilical vein endothelial cell(s)

ICAM-1 - intercellular adhsion molecule type-1

ICI 182,780 - the pure estrogen receptors antagonist

IFNγ - interferon-γ

IL-1β - interleukin-1β

IL-8 – interleukin-8, the inflammatory cytokine

iNOS - inducible niric oxide synthase

IP3 - inositol trisphosphate

K^+ - potassium ion

KDR - kinase-insert domain receptor or VEGFR-2

LDL - low-density lipoprotein

L-NAME - N^G-nitro-L-arginine methyl ester

L-NMMA - N^G-monomethyl-L-arginine

LPS - lipopolysaccharide

MAPK - mitogen-activated protein kinase

MAPKAP-2 - MAPK-activated protein serine/threonine kinase-2

MC – medium complete (MCDB131, 15% fetal bovine serum, growth factors)

MCP-1 - monocyte chemotactic protein-1

MEC - microvascular endothelial cells

MIC – medium incomplete (M199-RPF, 10% charcoal-stripped serum)

MMP-1 – matrix metalloproteinase-1

MMP-2 – matrix metalloproteinase-2

NAD(P)H - nicotinamide adenine dinucleotide (phosphate) oxidase

NMDA - *N*-methyl-D-aspartate

nNOS - neuronal nitric oxide synthase

NO- nitric oxide

NO_2 - nitrite

NO_3 - nitrate

NOS - nitric oxide synthase

O_2^- - superoxide

$ONOO^-$ - peroxynitrite

OVX - ovariectomized

oxLDL - oxidized low-density lipoprotein

PAEC(s) - pulmonaty artery endothelial cell(s)

PAF – platelet-activating factor

PAI-1 – plasminogen activator inhibitor-1

PAK1 - p21-activated kinase-1

PGI_2 - prostacyclin

PI – propidium iodide

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

PI(3)K/AKT - phosphatidylinositol-3-OH kinase/serine-threonine kinase-B

PKA – protein kinase A

PMA - phorbol myristate acetate

PMN - polymorphonuclear leukocytes

PTP - permeability transition pore

ROS - reactive oxygen species

RPF – red phenol-free

RTK - receptor tyrosine kinases

SERCA - sarcoplasmic reticulum Ca^{2+} ATPase

SERM - selective estrogen receptor modulators

SMC(s) - smooth muscle cell(s)

SOD - superoxide dismutase

Src - protein tyrosine kinase

Tek - tunica interna endothelial cell kinase

TMX - tamoxifen

TNFα - tumor necrosis factor-α

TRLEC(s) - simian virus 40-transformed rat lung endothelial cell(s)

uPA – urokinase type plasminogen activator

uPAR - urokinase type plasminogen activator receptor

VCAM-1 – vascular cell adhesion molecule type-1

VDAC - voltage dependent anion channel, porin

VEGF - vascular endothelial growth factor

VEGFR-1 (flt-1) - vascular endothelial growth factor receptor-1

VEGFR-2 (KDR)(flk-1) - vascular endothelial growth factor receptor-2

VEGFR-3 – flt-4 or vascular endothelial growth factor receptor-3

VEGFRs - vascular endothelial growth factor receptors

VSM - vascular smooth muscle

VSMC(s) - vascular smooth muscle cell(s)

W - wortmannin, the PI(3)K inhibitor

WHI - Women's Health Initiative

WT - wild type

I. Introduction and Literature Review

I.1. Introduction

The incidence of cardiovascular disease (CVD) differs significantly between men and women, but the mechanism is not known. The incidence of cardiovascular diseases is low in premenopausal women and increases after postmenopause. Observational studies have shown the CVD is much lower in postmenopausal women receiving estrogen therapy (1-3). More than 40 observational studies suggest that women who take estrogen have a risk of coronary heart disease that is 35 to 50 percent lower than the risk among women who do not take estrogen (4;5).However, recent large randomized secondary prevention studies, the Heart and Estrogen / Progestin Replacement Study (HERS) (6), Estrogen Replacement and Atherosclerosis randomized trial (ERA) (7) and some other smaller studies (8-10), did not find a reduction of CVD with HRT.

The benefits from hormone replacement therapy (HRT) seen in large observational studies might be attributed at least in part to the differences between women who choose to take hormones after menopause and those who do not, including differences in the level of education, access to medical care and several other factors like healthier life-style (4;11). In the past three decades, a large portion of the prescriptions for HRT in postmenopausal women has been for its possible cardioprotective effects (12). Randomized trials have shown that estrogen therapy reduced plasma levels of low-density lipoprotein (LDL) by 14 % and increased plasma levels of high-density lipoprotein (HDL) by 8 %, changes that are known to be associated with a reduced risk of cardiovascular disease (CVD) (6;13).

The HERS (6) randomized trial assessed the efficacy of hormone replacement for secondary prevention of coronary heart disease (CHD). A total of 2763 postmenopausal women (mean age, 66.7 years) with established CHD were randomized to receive placebo or HRT, which was given as a combined regimen of estrogen plus progestin. After an average of 4.1 years of follow-up, there was no overall reduction of CHD events. An extended follow-up study up to 6.8 years of these women (HERS II), did not confirm the observed trend of fewer CHD in the hormone group than in the placebo group in the years 3 to 5 compare to more CHD events during the first year of HRT (14).

The findings of HERS and HERS II with regard to a lack of benefit of HRT in high-risk women were supported by findings of 2 randomized trials that assessed the effect of HRT on progression of atherosclerosis. The ERA trial conducted in 309 postmenopausal women with verified coronary artery disease randomly assigned to receive HRT or placebo showed that the progression of coronary atherosclerosis was unaffected by HRT after 3.2 years of follow up (7). In the Postmenopausal Hormone Replacement Against Atherosclerosis trial, 321 postmenopausal women with increased carotid intima-media thickness, which signifies a subclinical atherosclerosis, were randomly assigned to receive placebo or HRT. After 48 weeks, there was no difference between the placebo and active treatment groups in progression of atherosclerosis measured as a change in carotid intima-media thickness. Furthermore, they noted 1 death of intracerebral bleeding in the HRT group as a serious adverse event (15).

The HERS and HERS II findings for ischemic stroke among high-risk women were consistent with the results of the Women's Estrogen for Stroke Trial (10). The randomized trial was conducted in 664 postmenopausal women (mean age, 71 years)

with high risk of CHD. After 2.8 years of follow-up, estradiol did not reduce mortality or the recurrence of stroke in postmenopausal women with cerebrovascular disease. The retrospective analysis of The Coumadin Aspirin Reinfarction Study (CARS) database of 1857 postmenopausal women on HRT with a recent myocardial infarction showed an increased risk of cardiac events during follow-up (8).

The Women's Health Initiative (WHI) (16) enrolled 16608 healthy postmenopausal women. It was the first randomized trial to directly address whether HRT had a favourable or unfavourable effect on CHD incidence. The trial was stopped early based on health risks that exceeded health benefits over an average follow-up of 5.2 years.

A randomized trial for secondary prevention in postmenopausal women with diagnosed unstable angina tested the possible benefit of short-term estrogen therapy. However, the 21-day of estrogen alone or combined with progestine in addition to standard therapy did not alter electrocardiographic evidence of ischemia (17).

The effectivness of HRT was also reported on noncardiovascular end points. Epidemiological studies have consistently shown a decrease in the risk of hip fractures in estrogen users by about 25% (2).

The atheroprotective effects of estrogens were initially attributed principally to lipid lowering effects of estrogens (3;18). This, however, only explains part of the benefits (19). Recent data suggests that direct actions of estrogens on blood vessels contribute substantially to their cardiovascular protective effects (20).

Criticism of trials in the literature

Although for years ERT was prescribed for postmenopausal women to prevent CVD, conclusions of recent clinical trials for secondary prevention indicated possible negative consequences of estrogen replacement (7;10;14). In the HERS trial, the risk of stroke in women with known coronary artery disease was not reduced by HRT (21). This conclusion was supported by results of WHI study, a trial for the primary prevention of CVD (16). Estrogen therapy alone or combined with progestin was shown to increase risk of stroke (22;23).

The timing of HRT initiation in relation to the onset of menopause or to age is a key factor in the risk of coronary heart disease (24;25). The discrepancy between observational and recent randomized trials may be explained by the age at which treatment was initiated. This hypothesis is recently the subject of validation in a prospective analysis of existing data known as the "timing hypothesis" (26).

With the exception of WHI (16), the subjects of secondary prevention trials were women with established atherosclerotic disease. In WHI, the average age at entry was 63, the majority of women came from lower socioeconomic classes and had many CVD risk factors and because of that many of the women in WHI likely had underlying atherosclerotic disease (27).

Estrogen may have positive or negative cardiovascular consequences depending on the circumstances. Estrogen could protect women from CVD by speeding repair of injured endothelium and restoring an anti-atherogenic state (28). On the other hand, neovascularization as the pro-angiogenic effect of estrogen, which is common around

plaques could lead to plaque instability and rupture (29). In secondary prevention trials and the WHI, atherosclerotic plaques were likely well developed.

Another possible reason for the failure of HRT to prevent CVD in the randomized trials could be the type and dosing of HRT (30). Progestins, especially medroxyprogesterone decrease the beneficial action of estrogen on lipids (13). Indeed, estrogen alone seems to have less complication than the combination with progestins (27).

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 nitric oxide (NO), which may be both through genomic and non-genomic mechanisms (19).

Thus, estrogen effects through a vasoregulatory substance, the potent vasodilator NO which is constitutively secreted by ECs. Prostacycline (PGI_2) is an additional vasoactive compound. Remarkably, ECs also synthesize other vasoregulatory substances, such as endothelin-1 (ET-1), the most potent vasoconstrictor, and platelet-activating factor (PAF). It is evident that the endothelium plays a key role in regulation and trafficking of many cellular elements of the blood. Therefore, I included a short overview on this organ (31).

I.2. Role of Endothelium

The endothelium provides a structural barrier between the circulation and surrounding tissue. Endothelial cells (ECs) *in vivo* rest on a basement membrane, which attaches the EC to the blood vessel wall. *In vitro*, ECs synthesize and secrete several types of collagen, fibronectin and thrombospodin which are incorporated into the EC extracellular matrix (32). This subendothelial structure provides integrity, mechanical strength and elasticity to the vessel (33). The endothelium, formed by the ECs and the subendothelium, represents a relatively impermeable surface that limits passive transfer of cellular and fluid particles between the circulating blood and the body's tissue (32). ECs line vessels in every organ system and regulate the flow of nutrient substances. They also secrete mediators that influence vascular hemodynamics in the physiologic state. With its overall content of 1×10^{12} cells on a surface area of 1000 m^2 and weight in excess of 100 g (32), the endothelium is a location for diverse biologically active molecules such as membrane-bound receptors for growth factors, coagulant and anticoagulant proteins, low-density lipoprotein (LDL) transporting particle, hormones and the blood cells themselves. Quiescent ECs maintain an active antithrombotic surface and are a site for secretion and uptake of vasoactive substances. Consequently, the endothelium plays an important role in regulating blood flow (34).

EC heterogeneity

The endothelial lining inside of blood vessels is formed structurally and functionally from a heterogeneous population of cells. This may account for the fact that many human vascular diseases are exquisitely restricted to specific types of vessels. For example, the susceptibility to atherosclerosis differs between arteries and veins. A marked regional variation in disease expression is well known in spite of there being systemic risk factors such as inherited disorders of lipoprotein metabolism, which should affect all EC. ECs from distinct regions of the artery are exposed to different flow patterns. The majority of the arterial endothelium is exposed to undisturbed laminar flow from unidirectional shear stress. In contrast EC in arterial curvatures or bifurcations are exposed to nonuniform laminar or turbulent flow (35). These differencies in flow pattern contribute to the heterogeneity of ECs; in (34). ECs of arteries are exposed to a similar composition of circulating mediators whereas the composition of the blood contacting venous ECs varies. The capillaries experience even more intensive variations because they are separated from organ tissues by a single layer of endothelial cells and basal lamina with an occasional pericyte applied to the outer surface (36-39). The microenvironement is also important for maintaining endothelial heterogeneity (40).

Atherosclerosis is a disease in which endothelial heterogeneity plays an important role in its development. The analysis of atherosclerosis-related genes revealed that stimulation with oxLDL in coronary artery ECs highly induced genes that regulated cellular proliferation, adhesion and apoptosis compared with human saphenous vein EC. In contrast, stimulation with classical proinflammatory cytokines IL-1β and TNFα caused

similar gene expression response of arterial and venous ECs. These data support a model of site-specific atherosclerosis and suggest that different gene expression could contribute to differences in atherosclerotic disease susceptibility (41).

The endothelium not only mechanically divides the circulation and surrounding tissue, but it also secrets important vasoactive mediators. Vasodilators, such as NO and PGI_2, as well as vasoconstrictors i.e. ET-1 and PAF, contribute to the regulation of vascular hemodynamics (34).

I.3. Estrogen – Mechanism of Action

Synthesis, sources, transport and metabolism of estrogens

The naturally occurring estrogens 17β-estradiol (E2), estrone (E1) and estriol (E3) are C18 steroids derived from cholesterol. Cholesterol is bound to lipoprotein receptors, taken up by steroidogenic cells and moved to the sites of steroid synthesis (42). These intracellular cholesterol transfers are facilitated by intracellular proteins such as the sterol carrier protein-2 (43). Transfer of cholesterol from the cytosol to the mitochondrial inner membrane, insured by steroidogenic acute regulatory protein (44), initiates the cleavage of cholesterol by cytochrome P450 enzymes. Aromatisation is the last step in estrogen formation. In hydroxylating reactions, estrone and estradiol are formed from their obligatory precursors androstendione and testosterone, respectively.

Endothelial cells are exposed to estrogens from numerous endogenous sources. In premenopausal women, the theca and granulose cells of the ovaries produce 17β-estradiol, the major circulating estrogenic compound (42). According to the "two-cell" theory of estrogen synthesis, the theca cells secrete precursor androgens that diffuse to the granulosa cells to be aromatized to estrogens (45). Estrogens and androgens however, could be synthesized by both of these types of cell (46). It also has been identified that there are a large number of other circulating estrogens and estrogen metabolites of these. Estrone and estriol are primarily formed in the liver from estradiol (42).

Circulating estradiol concentrations are low in adolescent girls, ranging from 55 to 128 pmol per liter (47) and estradiol production and serum concentrations are lowest premenstrually. In adult women, serum estradiol levels in the early follicular phase can

be as low as 73 – 367 pmol/L whereas the midcycle peak can read 550 – 2753 pmol/L and may rise to nearly 100 nmol/L during pregnancy. In postmenopausal women, serum estradiol concentration decreases to that found in adult males and ranges from 37 to 110 pmol/L (48). Most of the estradiol in these women is formed by extragonadal conversion of testosterone, and estrone becomes the predominant estrogen in this age group. The level of estrogen synthesis in peripheral tissues depends on the local expression and activity of aromatase and increases as a function of age and body weight (42). In the serum, estradiol reversibly binds to sex-hormone-binding globulin, a β-globulin (49), and with less affinity to albumin.

Lipoidal estrogens are fatty esters of estrogens that comprise a separate class of steroid hormones (50). They are found predominantly in adipose tissue. They are synthesized in blood, where they circulate and associate with lipoproteins, and mainly with HDL (51). However, serum estradiol can be transferred to LDLs by an unknown carrier mechanism. Lipoidal estrogens may serve as a potentially relevant steroid reserve; they also lower the concentration of estradiol necessary to inhibit oxidation of LDLs in vitro (52).

Estrogen Receptors

The steroid hormone estrogen exerts its effects by modifying gene expression through the activation of estrogen receptors (ERs).

There are two estrogen receptors, estrogen receptor α (ERα) and estrogen receptor β (ERβ), both of which are members of the superfamily of steroid hormone receptors. ERs are transcription factors that alter gene expression when they are activated (53;54). Estrogen receptors α and β have considerable homology in their DNA-binding domain

(96%) and in the hormone-binding domain (HBD)(53%) which includes the site of the hormone-dependent transcriptional-activation function. The amino-terminal domain of the ERs, which contain ligand-independent transcriptional-activation function, is unique for each ER. Estrogen-estrogen receptor complexes form homodimers and bind to specific gene sites. In addition, ERs α and β can form heterodimers with each other (55).

Identification of estrogen receptors

Decades ago, numerous investigations by Jensen and collaborators established that estrogen-responsive tissues such as uterus, vagina, anterior pituitary and mammary tumors contain characteristic components which show a high affinity for E2 (56). These E2-binding substances of target tissues have been called "estrogen receptors" or "estrophiles" (57). Strong but reversible association of hormone with receptor, without chemical transformation of estradiol molecule, appeared to be a primary step in the uterotrophic process that could be specifically blocked by E2 inhibitors (58;59). Jensen determined that the interaction of E2 with (rat) uterine tissue involved a two-stage mechanism: the hormone associated spontaneously with an extranuclear "uptake" receptor. The "small portion" of this receptor then transferred the E2-receptor complex to the nucleus (60).

Estrogen generally was initially considered as a steroid hormone that acted on tissues relevant to gonadal function where ERs were characterized. The human ER(α) was cloned from a cDNA expression library produced from the breast cancer cell line MCF7 (61). A novel nuclear receptor, the clone 29 was isolated from a rat prostate cDNA

library (62). Clone 29cDNA encoded a novel rat ER, which was present in the secretory epithelial cells of the rat prostate and also in granulose cells of the rat ovary. It was named ERβ to distinguish it from the previously cloned ER, which was subsequently named ERα. Further characterization of the genomic structure and chromosomal localization of human ERβ gene confirmed that the two ER genes were independent and did exist in the human (63). ERβ had subtle and important structural functional differences from ERα (62;64-67).

Tissue distribution of ERs

Tissue distribution of ERs in the wild type (WT) and ERα knockout (ERKO) mouse is established in detail by Couse and al. WT uterus and mammary glands have the greatest concentration of ERα, whereas WT ovary possesses the highest level of ERβ. In cardiovascular tissues, ERα mRNA is found in the heart and aorta of both genders, whereas ERβ is only minimaly detectable in the heart (68). In cardiovascular tissue of humans, others have found that ERα is expressed in vascular smooth muscle cells (VSMCs) from female coronary arteries (69) and in VSMCs from human saphenous vein (70) as well as in human endothelial cells (71-73). ERβ is found in many non-vascular tissues (prostate, uterus, lung, brain) (62;64) but also in the blood vessels of normal mice and rats (74;75). ERβ is still present in the blood vessels of mice in which the gene for ERα has been disrupted (74). Functional ERs are found in rat myocardial cells, and E2 exposure modulates ER-gene expression in these cells (76). Expression of ERα is much lower in human atherosclerotic vessels than in normal arteries, suggesting a role for the

lack of ER in atherosclerosis (69). It was shown that ERβ has a greater binding affinity than ERα in rat vascular tissues; this could allow it to play a vasculoprotective role (77). It is evident that ERα and ERβ have been identified in ECs but reports are inconsistent as to their presence. In our own studies, we were able to identify both ERα & ERβ in HUVEC, but also found variability in expression among cell culture to detect both ERs at the level of mRNA (see *Fig. 1 & 2 in the Chapter V of General discussion*). Recently, dynamic alterations by E2 in ERα and ERβ protein expression and in ERα-gene transcription in EC have been observed (78;79).

Functional role of ER

As to the function of ERs in the vasculature, studies on mice deficient in ERα (74) or ERβ (80) have shown that either of the two known ERs is sufficient to protect against vascular injury upon the effect of estrogen. These findings suggested two hypotheses: first, that ERα and ERβ were able to complement one another such that each receptor alone was sufficient to mediate the vascular protective effects of E2. The second hypothesis anticipated that the vascular protective effects of E2 were mediated by an ERs-independent pathway. To test these hypotheses, the effect of E2 on the response to vascular injury was examined in OVX, wild type (WT) or double ERα,β-knockout $mice_{Chapel\ Hill}$ ($ER\alpha,\beta KO_{CH}$) treated for 21 days with E2 or placebo and subjected to unilateral carotid artery injury resulting in endothelial denudation. E2 replacement in the WT mice significantly inhibited the injury-induced increase in medial area. In contrast, in the $ER\alpha,\beta KO_{CH}$ mice, E2 treatment did not inhibit the increase in medial area of injured

arteries. The increase in medial area of injured artery was similar to that measured in placebo-treated WT mice. Loss of the protective effect of E2 on the medial-area of carotid artery in ERα,βKO$_{CH}$ mice supported the concept that ERα and ERβ mediated this effect of E2 and could functionally complement one another. Surprisingly, however, the inhibitory effects of E2 on VSMC proliferation in the ERα,βKO$_{CH}$ mice still occurred as well as the E2-induced increase in the weight of uterus in ERα,βKO$_{CH}$ mice. The mechanism was unknown (81).

The abovementioned study did not answer the question whether E2 inhibition of VSMC proliferation and increase in the weight of uterus in ERα,βKO$_{CH}$ mice was caused by a receptor-independent mechanism, by an unidentified receptor responsive to E2 or by residual activity of the ERα splice variant reported previously in the parental ERαKO$_{CH}$ mice. To distinguish between these possibilities, the same group of investigators undertooke another study of mice in which ERα has been fully disrupted, ERαKO$_{Strasbourg}$ (ERαKO$_{St}$). They showed that after vascular injury in OVX ERαKO$_{St}$ mice, E2 had no detectable effect on any measure of vascular injury, including medial area, proteoglycan deposition or smooth muscle cell proliferation. These data demonstrated that ERα was responsible for the protective effects of E2 on the response to vascular injury in this study (82).

In summary, both ERα and ERβ are expressed in endothelial and VSMC and both receptors can mediate the protective and harmful effects of estrogen against acute vascular injury (83).

Estrogen receptors localization

Estrogen receptors classically act as nuclear transcriptional factors and modulate gene expression (53;54). However, recent evidence suggests that a nongenomic action of estrogen may have an important role (84;85).

Pietras and Szego (86;87) originally described an E2-binding protein in cell membranes that triggered the rapid generation of cAMP. Subsequent work indicated that E2 activated cell membrane based signaling, such as intracellular calcium influx (88), phospholipase C activation (89) and inositol trisphosphate (IP3) generation (90). These effects of E2 required E2 binding to ERs that were localized in the cell membrane. Moreover, these signaling events were likely to arise from the activation of G-proteins by E2. This was directly shown in transiently transfected chinese hamster ovary (CHO) cells with either ERα or ERβ. E2 bound to CHO- ERα or CHO- ERβ and activated Gαq and Gαs proteins in the membrane which led to rapid production of IP3 and stimulation of cAMP activity. (91).

ERs translocate to the plasma membrane where the receptors are attached to the bilayer facing to the cytoplasm within plasmalemmal caveolae (92-94). Data so far support the idea that membrane-localized ERs are very similar to the classical nuclear ERs. A variety of antibodies directed against multiple epitopes of the nuclear ERα identified an endogenous membrane protein in several cell types mainly originated from tumors (95;96). Expression of cDNAs for either ERα or ERβ in Chinese hamster ovary (CHO) cells which have no endogenous ER resulted in the detection of both cell membrane and nuclear ERs, indicating that the two ERs could originate from a single transcript and have

near identical affinities for E2 (91). More recently, endogenous ERα of 67 kDa and ERβ of 54 kDa have been identified in the caveolae and cell membranes from ECs using antibodies against the classical nuclear ERα or ERβ (92;97).

ERα and ERβ were not detected in the cell membrane or nuclei of ECs from double-knockout ERα/ERβ (DERKO) mouse in contrast to wild type ECs that endogenously expressed both ER isoforms at both locations (98). This led to a conclusion that the genes that code for the classical ER isoforms produced the membrane ER proteins. Whether nuclear and membrane ER proteins are identical could not be determined from this approach.

It has been proposed that an orphan G-protein-coupled receptor called GPR30 may be involved in physiological responses specific to hormonally responsive tissues (99) and that cellular expression of GPR30 is sufficient for E2-induced activation of Erk1/2 involved in nongenomic signaling by E2 (100). GPR30 has been shown to respond to E2 at the plasma membrane (100), or in the endoplasmatic reticulum (101). A recent study has shown a weak binding of GPR30 by sex steroid (102) whereas stable transfection of MCF7 cells with antisense oligonucleotides to GPR30 did not affect E2 activation of ERK nor cell proliferation in these breast cancer cells (103). E2, however, stimulated the growth of keratinocytes through the binding of E2 to GPR30 and subsequent induction of cAMP/protein kinase A (PKA) pathway (104). Previous study also reported that E2 generated cAMP signal via GPR30 in breast cancer cells (105).

To address these controversies, Razandi and collaborators again used the model of DERKO mouse. They found, that ERα/ERβ-deleted ECs isolated from mouse brain capillaries, had no functional E2-binding proteins. There was no binding of labeled E2 in

the whole cell, membrane or cytoplasmic fractions of DERKO mice ECs in contrast to strong binding of E2 in WT ECs. They determined also whether E2 could activate rapid signaling in the DERKO ECs. Whereas E2 induced cAMP, ERK and AKT phosphorylation as a measure for PI(3)K activity in WT, DERKO mice lacked these functions, indicating that DERKO ECs did not contain functional estrogen-binding protein. In EC of wild-type mice E2 stimulated thymidine incorporation by a MAPK-dependent mechanism, but thymidine incorporation did not occur in DERKO ECs incubated with E2. However, both DERKO and WT mice had comparable GPR30 protein expression. These results indicated that classical ERα or ERβ were necessary for activation of rapid signaling by E2. To examine the potential role of GPR30 for E2 signaling, investigators used ER-positive MCF7 breast cancer cells that produced GPR30, and ER-negative breast cancer cells (SKBR-3) that expressed the orphan GPR30. E2 mediated the rapid multiple signals on ERK, PI(3)K, cAMP and Ca^{2+} only in MCF7 cells but not in ER-negative SKBR-3. When the GPR30 in MCF7 cells was silenced, E2 still activated the rapid signaling to cAMP, ERK, PI(3)K and Ca^{2+}. E2 induced thymidine incorporation in MCF7 and this was inhibitable by ICI 182,780, but was unaffected by GPR30 silencing. The same investigators confirmed in MCF7 breast cancer cells transfected with small interfering RNA that the membrane and nuclear receptors were the classical ERα. In summary, E2 did not require GPR30 for rapid signaling in MCF7 cells but did require a classical ERα (106).

Plasmalemmal caveolae and ERs

ERs are localized to plasmalemmal caveolae of the plasma membrane. Endothelial caveolae are flask-shaped vesicular invaginations of the plasma membrane and are functionally involved in cellular transport mechanism (107). Caveolae are plasma membrane assemblies of glycosphingolipids and cholesterol that are associated with specific molecules, including signaling proteins. Often they contain the protein caveolin (108). Caveolin-1, the caveolae structural coat protein positively regulates the ER signal transduction.

It has been shown that coexpression of ERα with the caveolin-1 resulted in translocation of ERα to the nucleus and even prevented tamoxifen mediated inhibition of ERα signaling (109). Colocalization of ERα with the caveolae structural coat protein, caveolin-1 associated also with eNOS. Thus the binding of ER to caveolae was important in activation of endothelial nitric oxide synthase (eNOS). Calcium and the muscarinic cholinergic agonist carbachol promoted the dissociation of eNOS from caveolin in both bovine aortic endothelial cells (BAEC) and COS7 cells (110). It was primarily within caveolae where E2 activated eNOS after binding to ER (92-94).

It is known that notably in the vasculature prevail rapid, non-genomic effects of E2 (19;111;112). The short-term effects of E2 on eNOS stimulation and the enhancement of NO bioavailability in ECs (113) through ERα (114) is also known. The importance of caveolae for the rapid stimulation of eNOS by E2 through ER was shown explicitly by Chambliss et al.

They investigated the subcellular localization of interaction between ERα and eNOS. They used isolated EC plasma membranes to show that E2 in a dose-dependent manner increased eNOS basal activity; the effect at 30 minutes indicated that this was likely non-genomic effect. ERα as well as caveolin-1 were strongly expressed in the caveolae fraction of cell membrane whereas noncaveolae membrane contained ERα only. Subsequent evaluation of eNOS activity stimulated by E2 revealed that eNOS increased only in caveolae fractions. The E2 effect was efficiently abrogated by a pure ER antagonist ICI 182,780. Moreover, this process did not require Ca^{2+}/calmodulin (CaM) and eNOS cofactors. Thus this study clearly showed that E2 causes ER-dependent activation of eNOS linked to caveolin-1 in EC plasma membrane (92).

In another study the same group of investigators provided evidence that after E2 stimulation, endogenous ERβ mediated nongenomic eNOS activation in both, intact endothelial cells and isolated plasma membranes as well as caveolae membrane fractions (97).

The exact location of estrogen hormone receptors as well as the exact mechanism of the nuclear translocation of estrogen-estrogen receptor complexes is not yet fully known. They are probably in an equilibrium distribution between the cytoplasm and the nucleus and the direction of their translocation is modified after ligand binding (42).

Genomic effects of estrogen

Steroid hormones in general affect the transcription of mRNA and subsequently, protein synthesis, protein translocation and transport and protein actions. These effects take place after a long term lag phase (112).

Genomic effects of estrogens require hours to days for a modification in gene expression to occur. In the classical ligand –dependent activation of ERs, free estrogen diffuses into the cell, binds to the ligand-binding domain of the receptor, which then dissociates from its cytoplasmic chaperones. A complex of estrogen-estrogen receptor then diffuses into the cell nucleus. These estrogen-estrogen receptor complexes bind to specific palindromic sequences of DNA called estrogen-response elements (ERE) as homodimers or heterodimers. The ligand-activated estrogen receptor (ER) acts as a transcriptional factor (115;116). For example, the gene for nitric oxide synthase (NOS) contains 11 copies of the half-palindrome EREs often associated with an Sp1 site (117;118). Estrogen may increase the bioavailability of NO in vessels by increasing the expression of the gene for the inducible form of NOS, an important vasodilator enzyme (119). 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 acetylcholine (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.

Non-genomic effects of estrogen

In the past few years, there is increasing evidence for membrane-bound steroid receptors based on the rapid actions of various steroid hormones. This process is insensitive to cycloheximide because it does not require a protein synthesis de novo. Steroid effect that occurs between few seconds and 1-2 min after steroid application are likely due to a nongenomic effect (112). Estrogen was shown in numerous studies to act independently of transcription on target cells (90). The rapid effect of estrogens was shown in osteoblasts and osteocytes (90;120), breast cancer cells (121) and cerebral cortical cells (122;123).

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. Estrogen receptor (ER) is one of steroid nuclear receptors, which was found to be phosphorylated on tyrosine *in vivo* (124). Tyrosine phosphorylation of ER induced proliferation of MCF7 breast cancer cells (125;126).

An important pathway for E2 action is the stimulation of extracellular-regulated kinase (ERK), a member of the mitogen-activated protein kinase (MAPK), which is rapidly activated by E2 as a result of proximal kinase Src/Ras/Erk activation. ERK activation via this cascade contributes to E2-induced proliferation (121;127), survival of MCF7 cells (128) but also activation of eNOS in pulmonary artery endothelial cells (PAEC) (114).

Indeed, rapid stimulation of tyrosine phosphorylation was observed in cells treated with E2 for only 10 seconds (126) to 2 minutes (121). Stimulation of protein tyrosine phosphorylation of MCF7 cells peaked at 10 seconds and declined toward the basal level 60 minutes after treatment. This action of E2 required its occupancy of the receptor (126). E2 in MCF-7 cells immediately and reversibly affected tyrosine phosphorylation of MAP-kinases, ERK-1 and ERK-2, and its upstream associated protein $p21^{ras}$. ERK-2 kinase activity was very low or absent in COS7 cells, which did not express ER thus its level was unaffected by E2 treatment. In contrast, when the COS7 cells were transiently transfected with human ER-encoding plasmid, the kinase activity was increased after E2 treatment (121).

Tyrosine kinase inhibitors attenuated the E2-induced rapid increase in eNOS activity, indicating that eNOS activation required tyrosine phosphorylation. In addition, E2 caused acute (within 5 minutes) activation of eNOS in cultured PAEC. This process did not require the classical nuclear effects of the hormone because it was unaltered by the actinomycin D, an inhibitor of gene transcription indicating nongenomic ER activation. Thus, the acute increase in eNOS activity by E2 through ER could have been caused by the rapid activation of signaling pathways involving S*rc*-related tyrosine kinases and MAP kinase (114).

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-phosphotyrosine monoclonal antibody, which interacts with a broad range of phosphotyrosine containing proteins).

Thus, tyrosine phosphorylation is a regulatory mechanism, which influences eNOS activity, subcellular trafficking and interactions with other caveolin-interacting proteins. This also shows the importance of protein-protein interactions in NO signal transduction (129).

AKT phosphorylation

An additional signal transduction pathway originating from the membrane has been identified as being rapidly responsive to growth factors and E2. This effect is rapid and is thought to be primarily non-genomic. PI(3)K/AKT is considered one of the main signal transduction pathways to mediate this effects.

AKT/PKB is a serine/threonine kinase (protein kinase-B) that requires a functional phosphatidylinositol 3-kinase to be stimulated by insulin or by other growth factors. It plays a role in protecting cells from apoptosis (130). The conditionally active version of AKT fused into the HBD of a mutant murine ER, acutely stimulates the phosphorylation of a protein, which regulates protein synthesis (PHAS-I) (131). It was shown that NO production by EC in AKT-dependent manner was induced by simvastatin, a 3-hydroxyl-3-methyl coenzyme-A reductase (HMG-CoA) inhibitor. BAEC stimulated for 1 hour with simvastatin showed an increase in NO accumulation without a detectable change in the concentration of eNOS protein (132).

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 (133). Rapid stimulations of EC by vascular endothelial growth factor (VEGF) (134) and by E2 (135-138), activated PI(3)K and AKT kinase-induced downstream eNOS activity (92;93;114;135-138) and subsequently led to release of NO. PI(3)K/AKT phosphorylated human NOS on serine 1177 in response to shear stress (139) or VEGF (134). In ECs, the activation of AKT kinase by E2 and the generation of NO were inhibitable by ER specific inhibitor ICI 182,780. In vivo model of muscle injury following ischemia-reperfusion, this pathway was responsible for the ability of E2 to prevent leukocyte accumulation (136). Haynes et al. in permanently established ECs EA.hy 926[1] (140) demonstrated that E2 rapidly within 5 minutes activated PI(3)K/AKT pathway and phosphorylated AKT kinase, as well as eNOS on the specific serine 1177. This process did not require a rise in free intracellular Ca^{2+}. Moreover however, by using a membrane impermeant E2 conjugated to excess fatty acid-free bovine serum albumin (BSA) E2BSA, they showed that this pathway is activated by engagement of a surface ER (135).

The influence of nuclear ER in the effects of membrane ER has been tested also by E2BSA. NOS activity was found increased by treatment with E2BSA and was not altered by actinomycin D, an inhibitor of gene transcription. This indicated that the rapid E2 activation of eNOS did not require the classical nuclear effects of the estrogens. (137).

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[1] permanent human endothelial hybrid cell line established by fusing a HUVEC with a human lung carcinoma cell line A549/8

The PI(3)K/AKT pathway could be also activated by rapid effect of E2 through the proximal Src kinase with consequent enhanced NO release (141).

Zhang determined in COS7 cells that it was membrane localized ERα specifically that mediated the rapid E2 phosphorylation of MAPK signaling cascade (142). 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 E2 via mitogen-activated protein kinase-activated serine/threonine kinase-2 (MAPKAP-2 kinase) in primary cultures of BAEC. E2BSA within 10 minutes induced activation of the p38β member of the MAP-kinases and led to the activation of MAPKAP-2 kinase and the phosphorylation of Hsp27 which reduces endothelial cell permeability (143).

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. Steroid hormone receptors are their target molecules (144). The interaction of the ER ligand-binding domain with Hsp90 has been well characterized (145). Nonetheless, the role of Hsp90 in E2-mediated activation of eNOS is still unclear at present (146). Russell et al. showed that E2 promoted the association of Hsp90 and eNOS suggesting that Hsp90 may play an important role in the rapid, ER-mediated modulation of eNOS activation by E2. More, this activation appeared to significantly reduce the calcium-calmodulin dependence of the enzyme (147). Simoncini at al., however, reported that Hsp90 disrupted the interaction between ERα and the p85α regulatory subunit of PI(3)K (136), while Garcia-Cardena et al. demonstrated

that in endothelial cells eNOS not only existed in a heterocomplex with Hsp90 but Hsp90 directly activated eNOS in response to growth-factors such as VEGF(148). Research works of others supported the finding that E2 treatment produced rapid Hsp90-eNOS associatiation and activation in ECs. For instance, the geldanamycin, a specific inhibitor of Hsp90, markedly reduced the E2-induced vasodilatation after short-time incubation. ICI 182,780, an ER pure antagonist also blocked the vasorelaxation demonstrating that non-genomic effect of E2-ER through eNOS activation was coupled to Hsp90 (149).

I.4. Estrogen and Endothelium

Actions of estrogen in human and animals

In healthy vasculature, the endothelium releases nitric oxide (NO) in response to a variety of stimuli, such as acetylcholine (Ach), thrombin, substance P, calcium ionophore A23187, bradykinin, leading to vasodilatation (150). The same stimuli, however, in diseased blood vessels with dysfunctional endothelium where the release of NO is attenuated, cause a direct stimulation of smooth muscle contraction and subsequent paradoxical vasoconstriction (150). Estrogen acutely enhances vasorelaxation via both endothelium-independent (151;152) and endothelium-dependent (84;153-156) pathways. These effects are non-genomic as they do not involve changes in gene expression. E2 at supraphysiologic concentrations, could inhibit the influx of extracellular calcium (Ca^{2+}) into VSMC through an effect on cell membranes and/or L-type Ca^{2+} channels (157;158). At physiological levels, the principal acute effect of E2 on ion channels is to open vascular smooth muscle (VSM) calcium-activated potassium channels, which occurs via NO/cyclic guanylate monophosphate (cGMP)-dependent pathway (159-161). The released NO from intact endothelium represents a potent vasculoprotective system (150).

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 (19). A direct vasodilating effect of estrogen at pharmacological doses was observed on the rat tail artery (162). Acute estrogen exposure caused, within 20 minutes, an endothelium-dependent coronary dilation in female primates (156). Also,

acute administration of estrogen produced a rapid endothelium-dependent dilation of normal vessels and atherosclerotic arteries, which is believed to be dependent on the release of NO (19;112;163). Estrogen administration also prevents coronary arterial constriction in response to Ach in post-menopausal women (164).

In postmenopausal women with coronary artery disease, acute administration of E2 causes a vasodilatation of the coronary artery within 20 minutes of exposure. This effect is not apparent in men of a similar age with the same pathological condition (164). Sublingual administration of 1 mg of E2 to postmenopausal women produces an acute delay of exercise-induced myocardial ischaemia, which provides indirect evidence for a vasodilatory effect of estrogen in postmenopaual women (165). The mechanism by which estrogen appears to inhibit the development of atherosclerosis in females is through the increased formation of NO in vascular endothelium.

Long-term vascular effects of estrogen on NO in humans

A natural model for observation of effects of endogenous E2 or its depletion on vascular function provided the onset of **menopause**. The brachial artery function measured as flow mediated NO-dependent and endothelium-dependent vasodilatation after reactive hyperemia, was significantly reduced in either postmenopausal group, with HRT or without that substitution, compared with premenopausal women. Long-term combined oral HRT was without beneficial effects on endothelial vasomotor function in healthy postmenopausal women (166). Similarly, determinations of forearm blood flow (FBF) constrictor responses to NOS antagonism with N^G-monomethyl-L-arginine (L-NMMA),

which also evaluated vascular NO activity, revealed greater constriction in premenopausal women compared to that in postmenopausal women and males. In contrast, vasodilator responses to glyceryl trinitrate (GTN) were similar between groups, indicating comparable VSM responses to NO. Two weeks of estrogen replacement therapy (ERT) restored vascular NO activity to premenopausal levels (167).

The **menstrual cycle** provided also a natural model for studying the effects of endogenous E2 on endothelial function in humans. Indeed, flow-mediated vasodilation (FMD) of brachial artery paralleled the levels of serum E2 and nitrite/nitrate in young healthy females (168). A study of 28 eumenorrheic women found FMD by 36% greater during the E2 rise at mid-cycle than during the follicular phase. In luteal phase, when E2 but also progesterone was elevated, vasodilatation of brachial artery was similar to that found in follicular phase. Since the investigators found a negative correlation between progesterone levels and FMD, this suggested that progesterone antagonized the beneficial effects of E2 on vascular reactivity (169). The endothelium-dependent vasodilation of brachial artery, which varied during the menstrual cycle, and correlated with the levels of endogenous E2, was reported in the study that included also male subjects. Their FMD was comparable to that in female subjects only in phase when serum levels of E2 and progesterone were similar in both sexes (170). The fact that vascular responses in resistance vessels change during the menstrual cycle in women were shown in another study of healthy women. At a time of high E2 levels during the mid-cycle, the endothelium-dependent FMD to bradykinin was enhanced, whereas response to vascular smooth muscle dilator GTN were unchanged. Moreover, noradrenaline-induced vasoconstriction was enhanced during the high E2 phase of the cycle. The balance of

these two opposing actions, if applied on exogenous E2, may determine the beneficial or harmful effects of ERT in postmenopausal women (171). A protective role of endogenous E2 was assessed in a group of women subjected to surgical menopause by bilateral ovariectomy for uterine leiomyoma. Endothelium-dependent vasodilatation determined by FBF to acetylcholine (Ach) was significantly reduced after ovariectomy, but restored by exogenous E2 administered for 3 months as ERT. The response to a NO donor, sodium nitroprusside, however, remained unchanged suggesting that acute E2 deprivation led to selective impairment of endothelium-dependent vasodilatation (172). The enhanced endothelium-dependent vasodilatation observed in mid-cycle with greater E2 abundance correlated with higher serum levels of the NO metabolites nitrate (NO_3) and nitrite (NO_2) and greater exhaled NO (168;173). The mid-cycle peak and changes in NO production with the cyclical hormonal changes in healthy women were confirmed also by others (174).

The administration of **ERT** or **HRT** demonstrated that endothelium-dependent vasodilatation of the brachial and coronary arteries were enhanced after ERT in postmenopausal women. For example the 9-week ERT in a group of women in menopause (average age 55 yr.) with mild hypercholesterolemia, improved flow-mediated endothelium dependent vasomotion (154). In a group of postmenopausal women with early atherosclerosis ERT was associated with an attenuation or reversal of the vasoconstrictor response to Ach, suggesting a normalization of endothelial-dependent vasodilator capacity (175). ERT also caused elevations in plasma NO and NO metabolites NO_2 and NO_3 in postmenopausal women taking E2 for 2 years (176). The 6 months of ERT in a group of postmenopausal women (average age 53 yr.) increased the

ratio of NO to endothelin-1, but also decreased the LDL cholesterol levels and increased the HDL cholesterol levels (177). Furthermore, the 6 months of HRT and also raloxifene therapy in postmenopausal women reported not only increased NO_2/NO_3 levels and the ratio of NO to endothelin-1, but the flow-mediated, endothelium-dependent vasodilatation of the brachial artery was enhanced in the group of HRT and raloxifene compared to that of placebo, whereas endothelium-independent responses to a NO donor nitroglycerin were not altered (178). Similarly to the findings noted in variations in endothelial vasodilation during the menstrual cycle in instances of the elevated progesterone (169), the addition of progestine in postmenopausal HRT appeared to lower the effects of estrogen on endothelial NO production (173;176). In contrast of these observations, a study on older postmenopausal women (mean 76 years) receiving ERT showed decrease in lipids and LDL cholesterol levels, while HDL cholesterol increased. The addition of medroxyprogesterone to the replacement regimen did not reduce the beneficial effect of ERT vs. combination HRT (179). Long-term (6 months) treatment with high dose estrogens was associated with enhanced arterial reactivity in genetic males and this was at least partially mediated through endothelium-derived NO (180). The postischemic vasodilation of brachial artery was compared in 4 groups: in postmenopausal women on ERT for at least 3 years or in postmenopausal women without ERT for 10 years, in healthy young women and in aged matched men. The examination of vascular reactivity differences between groups showed that premenopausal women had greater postischemic vasodilation of the brachial artery then men and postmenopausal women without ERT. In addition, postmenopausal women with ERT had markedly

greater postischemic vasodilation than postmenopausal women without ERT but also than that in men (181).

In summary, the prolonged exposure to exogenous estrogens has positive effects on the availability of endothelium-derived NO, which correspond to those observed with altered endogenous estrogen levels with regard to long-term modulation of eNOS function by estrogens in humans (182).

Short-term vascular effects of estrogen on NO in humans

The evidence of short-term effect of E2 on eNOS function in humans was reported in several studies. The basal coronary vasomotor tone was altered in a group of 22 postmenopausal women, when ethinylestradiol (EE2) induced an increase in coronary flow, a decrease in resistance and an increase in epicardial cross-sectional area. Administration of placebo had no effect on these parameters. EE2 also attenuated paradoxical coronary vasomotor responses to Ach in these women when administered 15 minutes before Ach (183). Acute intracoronary administration of E2 did not affect basal coronary artery diameter, blood flow, or resistance, but there was greater vasodilatory response to concomitant infusion of Ach with E2. The reduction in coronary resistance from baseline in response to Ach was also potentiated by E2. The effect of E2 on coronary dynamics was most prominent in women with the most impaired responses to Ach in both large coronary conductance arteries and coronary microvascular resistance arteries. E2 had no effect on vasodilation with sodium nitroprusside, an endothelium-independent vasodilator, demonstrating that the hormone rapidly modified the availability of endothelium-derived NO (184). Physiological levels of acute (20 minutes) infusion of

E2 selectively potentiated endothelium-dependent but also endothelium-independent brachial artery vascular responses in postmenopausal women with risk factors for atherosclerosis and evidence of impaired vascular function (84). In the study on the coronary circulation of older, estrogen-deficient women who had an evidence of atherosclerosis, a rapid, 15 minutes intravenous infusion of E2 also decreased the intensity of Ach-induced epicardial coronary artery vasoconstriction. However, these responses were prevented by L-NMMA, an eNOS antagonist; E2 no longer potentiated the effects of Ach on coronary flow dynamics. Thus the mechanism of E2 effects on the vasculature were mediated by enhanced bioavailability of NO (85). In another study on older postmenopausal women (mean age 69.7 years), acute hormone therapy, however, did not reduce the established ischemia with unstable angina when added to standard anti-ischemic therapy (17). The differences between acute and chronic E2 effects were reported in the study on 33 postmenopausal women (average age 59 yers). Short-term ERT improved FBF and endothelium-dependent vasodilation in peripheral circulation. However, after 3 weeks of transdermal E2 administration, these parameters were unchanged from initial measurements obtained before acute administration of E2, probably due to the lower E2 plasma levels achieved with chronic ERT (185).

In the comparative study of postmenopausal women and similarly aged men with proven coronary artery disease, the acute (20 min) E2 administration modulated Ach-induced coronary artery vasodilations in female but not in male atherosclerotic coronary arteries suggesting that an enhancement of endothelium-dependent relaxation by natural estrogen from HRT may be important in postmenopausal women with established coronary heart disease (164). In contrast, two other studies in men on rapid (15 min) administration of

E2, augmented Ach-induced coronary blood flow (186) and improved coronary blood flow responses to an exogenous cold stimulus (187). A rare case of a disruptive mutation in the estrogen receptor gene in a 31-year-old man with premature coronary artery disease allowed studying the peripheral vasculature in the situation of estrogen resistance. The rapid vascular responsiveness to E2 was intact, but flow-mediated endothelium-dependent peripheral vasodilation was impaired. Since a vasodilation of the brachial artery after nitroglycerin was intact, this suggested a diminished capacity for endothelial NO production (188).

These observations indicated that besides long-term effects of E2 on the vasculature, also the short-term effects of E2 influenced the bioavailability of NO.

Long-term vascular effects of estrogen on NO in animal models

The long-term beneficial effects of E2 in **diseased vessels**, has been actively investigated in animal models. The physiological levels of E2 on the arterial response-to-injury were studied in OVX mice treated with E2 for 7 days and subjected to carotid injury. Analysis by quantitative morphometry showed increase in both intimal and medial area of controls, while E2 treated remained unaffected by injury (189). In study of carotid balloon injury in gonadectomized male and female rats on estrogen replacement for 3 days, E2 attenuated the resulting myointimal proliferation after vascular injury in both sexes (190). E2 also accelerated reendothelization after carotid artery balloon denudation in OVX rats, which were on ERT for 30 days. Functional endothelial recovery was manifested as an increase in NO production by carotid arteries from E2 treated rats vs.

placebos (191). E2 had capacity to modify vascular injury by promoting angiogenesis (192).

The impact of E2 on the function of atherosclerotic arteries was shown in OVX cynomolgus monkeys treated with E2 or placebo for 26 months. Atherosclerosis was induced by feeding an atherogenic diet. The E2 replacement reduced plaque extent in coronary arteries and modulated vasomotion of atherosclerotic coronary arteries of monkeys as determined by altered vascular responsivness to the endothelium-dependent dilator acetylcholine (193). The effects of combine HRT with addition of medroxyprogesterone to the regimen with E2, was examined on surgically postmenopausal cynomolgus monkeys with diet-induced coronary atherosclerosis. In the group treated with E2 alone, the endothelium-mediated dilation of coronary arteries was improved, while the addition of medroxyprogesterone diminished the beneficial effects of unopposed E2 on endothelium-mediated dilation (194). The treatment with E2 (3 weeks) of OVX hypertensive female rats preserved NOS resulting in a greater modulation of pressure-induced myogenic tone than in male rats and maintenance of NO-mediated vasodilations (195). In OVX rats, a chronic (1 yr) E2 treatment attenuated aging-related changes in vascular stiffening and permeability of carotid arteries (196).

E2 substitution has been shown also to positively influence models of **metabolic disorders**. The beneficial effects of E2 treatment (7 days) were observed in study on cholesterol-fed female rabbits. The catabolism of VLDL in these animals depended on the composition of VLDL. E2 treatment irreversibly removed cholesterol- and apolipoprotein E-rich VLDL in cholesterol-fed rabbits on ERT (197). Chronic treatment (90 days) with E2 prevented hyperglycemia-induced accelerated formation of

atherosclerotic lesion in male apolipoprotein E-deficient mice (198). Iliac arteries from OVX rats 6 month on ERT, demonstrated decreased glycoxidative damage and oxidative stress in the arterial wall. E2 treated rats also had decrease in plasma glucose level (199). The **role of ER** in the effects of E2 on the response to vascular injury has been addressed using genetically modified mice (74;80;81). For example, in one study on OVX, ERα-knockout mice with vascular injury, the 21-day ERT failed to prevent the injury-induced increase in the vascular media (82). Another way to show ER-mediation of E2 vasculoprotective effects was to use a pure nonselective ER antagonist ICI 182,780. This inhibitor blocked the vasculoprotective effect of E2 on neointima formation in OVX rats with carotid balloon injury on 2-week ERT (200).

The numerous research studies in animal models showed that long-term exposure to the E2 enhanced the capacity for **endothelial NO production**. For instance, the study on chronic E2 treatment (4 days) of OVX rabbits increased the endothelium-dependent responses in isolated femoral artery rings. Significant relaxations to Ach were obtained with lower concentrations in the E2-treated group than in the untreated group (201). On isolated thoracic aortae rings from male and female rats randomly chosen on different days of the estrous cycle was shown that the suppression of the contractile responses to phenylephrine by the endothelium were significantly greater in aortae isolated from female rats. Ach evoked significantly greater endothelium-dependent relaxation in aortae of female rats compared to male rats suggesting the augmented NO production in female rats (202). The significant association between the number of ERs and basal release of NO was shown in the aorta of mice. An estrogen receptor knockout (ERKO) male mice

was used, as it was shown that aorta from male mice contained more than twice as many E2-binding sites than in the female aorta. Indeed, the effective disruption of ER gene led to significant decrease in basal endothelial NO production in male ERKO mice. This change occurred in spite of unchanged circulating plasma levels of E2 compared with control male mice, suggesting that functional ERs and not the E2 plasma level are important in modulating endothelial NO production in mouse aorta (203). The influence of pregnancy and sex hormones, notably E2, on Ca^{2+}-dependent NOS was studied in the guinea pig. Both, pregnancy and E2 treatment increased the amount of mRNAs for eNOS and nNOS in skeletal muscle, suggesting that the increases in NOS activity resulted from enzyme induction by E2 (204). The long-term effects of E2 on eNOS expression have been demonstrated in studies of the uterine circulation in OVX nonpregnant sheep receiving E2 treatment for 3 days. E2 stimulated the eNOS activity in uterine arteries but not in renal arteries. To the greater NOS-enzymatic activity was related an enhanced NO-dependent vasorelaxation (205). On the similar models of OVX ewes uterine arteries was shown, that prolonged, but also acute E2 treatment caused upregulation of eNOS enzymatic activity (206;207). The chronic E2 treatment on eNOS activity in rat cerebral microvessels was studied on gonadectomized male and female rats with long-term (4 weeks) ERT. The E2 treatment increased the amount of eNOS protein in cerebral microvessels of both male and female gonadectomized rats, which indicated that exogenous or endogenous E2, but not testosterone caused this increase and may contribute to neuroprotective effects (208). The study of eNOS in cerebral microvessels of knockout mice demonstrated, that it was ERα responsible for mediation of E2 effects on eNOS upregulation. E2 treatment had no effects on diameter of cerebral arteries of

ERα knockout mice (209). The direct effects of E2 on porcine coronary arteries were demonstrated after long (18-22 h) exposure of isolated arterial segments from OVX pigs. E2 enhanced endothelium-dependent NO-mediated vasorelaxation in these arteries (210). In several studies on animals, the selective estrogen receptor modulator (SERM) raloxifene replaced the E2. For example, in OVX ewes the raloxifene significantly increased uterine and coronary blood flow within 6 and 24 hours respectively. These responses were partially mediated by NO (211). Raloxifene also prevented the decrease in eNOS activity caused by E2 deficiency in OVX rats (212). The effects of genistein, a phytoestrogen with a flavonoid chemical structure, paralleled to those of E2 in OVX rats. Animals were treated for 4 weeks with E2 or genistein. As assessed by vasorelaxation of aortic segments by Ach and by measurement of eNOS activity, the genistein supplementation reverted the endothelial dysfunction and enhanced the eNOS acivity to the same extent as did E2 treatment (213).

Short-term vascular effects of estrogen on NO in animal models

The rapid effect of E2 in the vasculature was explicitly demonstrated in the original study on OVX female cynomolgus monkeys fed an atherogenic diet. Before infusion of E2, intracoronary administration of Ach caused vasoconstriction, while after administration of E2, Ach caused coronary vasodilation. Dilator responses to nitroglycerin were unchanged before or after E2 administration. Thus, E2 had rapid effects on endothelium-mediated vascular responses of atherosclerotic coronary arteries (156). The increase in NO production following rapid effects of E2 on endothelium-dependent vasorelaxation

was shown on coronary arteries from OVX rabbits (214), on superior mesenteric arteries from OVX rats (215) and on aorta of female rats (216). However, in some studies the acute E2 administration induced a vasodilation NO-independently and endothelium independently (217;218). In one study was shown that direct vasodilating effects at pharmacological doses of E2 applied to male rat tail artery was mediated by its inhibitory effect on Ca^{2+} influx through voltage-dependent calcium channels in VSMCs (162). The E2 at pharmacological dose perfused through male rat hearts decreased heart rate and increased coronary flow in arteries precontracted with Ach, which suggested endothelium dependent vasodilatory effects of E2 (219). Physiological concentrations of E2 attenuated endothelin-1 induced vasoconstriction in female porcine coronary conductance and resistance arteries in vivo selectively through endothelin-receptor, which suggested a specific attenuating effect of E2 on endothelin-1 induced vasoconstriction (220).

In summary, studies related to endothelial NO-mediated vascular responses in humans and animals indicate that E2 causes both long-term upregulation of eNOS expression and rapid enhancement of eNOS enzyme activation.

I.5. Biology of Nitric Oxide

Main characteristics of NO

Nitric oxide (NO) is a small lipophilic and chemically unstable molecule. It has no charge and it is composed of seven electrons from nitrogen and eight electrons from oxygen. It contains an unpaired electron and is thus a free radical, but it is not highly reactive (221). The half-life of this free radical is only few seconds (222). A large source of NO synthesis is from the endothelial cell of the vascular wall. NO is synthesized not only by vascular EC but also can be synthesized by macrophages, neutrophils, hepatic Kupffer cells and brain tissue.

NOS isoforms

There are three, highly homologous isoforms of nitric oxide synthase (NOS). Two isoforms are constitutively expressed, although their expression may be modulated. nNOS (NOS1, neuronal) was initially thought to be expressed primarily in neurons; it was first isolated from rat and porcine cerebellum (223). eNOS (NOS3, endothelial), which is expressed in EC (224;225) of animal and human tissues (226), cardiac myocytes and blood platelets. The third isoform iNOS (NOS2, inducible) is normally not expressed but the expression is markedly induced by inflammatory cytokines such as TNFα, IL-1β, IFNγ and lipopolysaccharide (LPS). It is found in many eukaryotic EC of organs such as the liver, lung, kidney, heart, aorta and in cardiac cells (227). In EC, a variety of vasodilatory compounds including bradykinin, adenosine, acetylcholine (Ach), shear

stress, leukotriene D_4, histamine, dopamine and the calcium ionophore A23187, increase NO synthesis.

Synthesis of nitric oxide

The free radical NO is generated exclusively by the enzyme NOS. NO synthesis by NOS isoforms depends on the availability of L-arginine, BH_4 and NADPH (228). 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, of tetrahydrobiopterin (BH_4) and of free Ca^{2+}. The Ca^{2+} enters the cells (229) by mediation of glycine and glutamate of *N*-methyl-D-aspartate (NMDA) receptors. The enzyme NOS contains binding sites for heme and calmodulin (CaM), which are essential for the production of citrulline and NO. NO binds to iron in the heme and activates guanylate cyclase (GC). An increase in intracellular calcium stimulates the constitutive NOS to form NO from L-arginine (150;228). Electron transfer depends on interaction with the calcium-binding protein CaM, which in the case of nNOS and eNOS requires elevated intracellular free Ca^{2+} concentrations (>200 nM); essentially no activity is observed in the absence of Ca^{2+}/ CaM (223). CaM binding facilitates a conformational change of constitutive NOS to allow electrons to flow to the heme (230;231). By contrast, the basic, hydrophobic domain in iNOS monomers can bind CaM even at the trace levels of Ca^{2+} (232). iNOS enzyme possess CaM recognition sites, thus CaM is bound very tightly

to iNOS, with the binding unaffected by Ca^{2+} (233;234). For this reason, the intracellular Ca^{2+} concentrations were found not to play a role in the expression or regulation of the iNOS (235;236). NO synthesis can be abolished by L-arginine analogues such as N^G-monomethyl-L-arginine (L-NMMA), which inhibits both the production of citrulline and the increase in cGMP (237).

NO acts as a potent activator of cytosolic GC and elevates tissue cGMP levels (238). Under these conditions NO has been shown to stimulate cGMP-dependent protein kinases which leads to increased activity of Ca^{2+}-dependent (potassium) K^+ (K_{Ca}) channels (159-161;239;240). NO can freely diffuse across the cell membrane. NO diffuses to significant concentrations at distances relatively far removed from a single NO producing cell (241). NO rapidly accumulates locally. Endothelium can produce 10-40 fold more NO than necessary to activate GC but the majority of NO is lost to the vascular compartment (242).

NO plays a physiological role in local transcellular communication. 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 (243).

Chemistry of peroxynitrite

The vasoactive actions of NO synthesized by NOS can be substantially diminished *in vivo* by superoxide (O_2^-). Both NO and O_2^- are themselves weak oxidants but when

combine they produce peroxynitrite ($ONOO^-$) (244). The protonated form of peroxynitrite at physiological pH 7.4 is a potent oxidant with a half-life of approximately 1 second (245). A scavenger of O_2^-, superoxide dismutase (SOD), can enhance the biological activity of NO (246). However, the reaction between O_2^- and SOD occurs at a rate of 2×10^9 $M^{-1}sec^{-1}$ (247) whereas NO reacts with O_2^- at a rate of 6.7×10^9 $M^{-1}sec^{-1}$ (248). Nitric oxide, as measured by NO-selective microsensors (242), reacts with O_2^- faster than O_2^- - is scavenged by SOD or disintegrates by all other pathways what accelerates the formation of peroxynitrite (249). This means that the rate of production of either NO or O_2^- and the ratio of their concentrations, is more important in systems in which both radicals are produced than the actual concentration of either of these molecules.

Biological role of NO in the vasculature

Many investigators have shown the effect of NO release on vascular tone (250-253). Before it NO was identified as the substance emitted by the endothelium; it was called endothelium-derived relaxing factor (238). 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 (254). Isolated preparations of intact arteries, precontracted with norepinephrine, exhibit relaxation to the agonist Ach. This occurs through the action of muscarinic receptors on EC, which increase intracellular calcium, which activates NOS.

In the rat aorta, endothelium-dependent vasorelaxation produced by Ach is abolished by NOS inhibitors, indicating that NO mediates the vasodilatation.

NO mediated vasodilatation is diminished in the forearm arteries of patients with essential hypertension, and the decrease is greater as blood pressure increases. It also play a role in hypoxia induced dilation. As well, systemic vasodilatation evoked by hypoxia for this is prevented by L-NMMA (255). Thus, NO is a major factor regulating peripheral resistance.

One of the first observations of NO mediated vasorelaxation (256), was increased basal release of NO during vasodilatation in response to SOD, which is an O_2^- scavenger, and reduces the interaction of NO with O_2^- (257). Binding of NO by O_2^- results in an attenuation of NO signaling mechanism, such as those mediated through cGMP whose production from guanylate cyclase stops within seconds after NO is removed (258). In tissues, the interaction of NO with O_2^- results in the production of peroxynitrite (259).

The modulating effects of NO are evident by the release of NO from endothelium in response to shear-induced stress from turbulent blood flow, which stimulates the endothelium to synthesize NO. Released NO causes a local relaxation and increased diameter of the blood vessel as well as locally restores laminar flow (260). NO is thus a key element in the regulation of vascular tone.

A possible consequence of the loss of NO modulation of vascular tone is the development of atherosclerosis, which is predisposed to develop at sites of low turbulence. Atherosclerotic lesions are formed by oxidized lipoproteins that are scavenged by macrophages that accumulate in subendothelium. Atherosclerotic blood vessels have impaired responsivness to endothelium-dependent relaxation (261;262). Although the

mechanism that initially oxidizes lipoproteins is not defined, peroxynitrite was shown to modify LDLs into a form recognized by the macrophage scavenger receptor (263).

S-glutathiolation

The oxidative stress in a cell is a part of the normal cell physiology. It is caused by reactive oxygen species (ROS) such as superoxide radical O_2^-, hydrogen peroxide (H_2O_2), hydroxyl radical OH^-, and also nitric oxide radical NO^- and peroxynitrite radicals $ONOO^-$. The antioxidants are mainly glutathione (GSH), antioxidant enzymes like SOD, catalase and thioredoxins. Protein sulfhydryls may be oxidatively modified by S-thiolation, an important cell protective process necessary during oxidative stress. S-thiolation of proteins occurs primarily via GSH dependent trapping mechanism when protein-thiols (-SH) are oxidized to thiol disulfide bounds (264).

NO is an important modulator of muscle contraction through the formation of cGMP and its interaction with reactive oxygen species (ROS) which modify regulatory thiols in the sarcoplasmic reticulum (265). The simultaneous production of NO and O_2^- can promote the formation of $ONOO^-$, a strong oxidant which efficiently modifies protein thiols (266). There is also evidence indicating that ROS have signaling role in endothelial cells (267). ROS can signal intracellular events by the oxidation of cysteine residues and formation of disulfide (-S-S-) bounds (268). The change from thiols (-SH) to –S-S- bonds can produce a conformational change that results in protein-protein or protein-DNA interactions (269). Some reactions are irreversible and result in protein instability. A reversible process is oxidation of cysteinyl thiols by S-glutathiolation from thiol disulfide or from

direct oxidation of protein cysteinyl thiols. The reversible oxidation promotes the formation of mixed disulfide and prevents the irreversible thiol oxidation (270;271).

It has recently been shown that even the potentially toxic by-product of NO and O_2^-, $OONO^-$, regulates vessel mode (269). Cohen and coworkers found that NO-induced dilatation occurs by the production of low concentrations of $OONO^-$. The physiological vasodilatation is then subsequent to direct calcium (Ca^{2+}) ATPase stimulation of the sarco/endoplasmic reticulum (SERCA), which decreases intracellular Ca^{2+}. This is the process of reversible S-glutathiolation of the thiol of a cysteine residue on SERCA (269). Even basally released NO causes S-glutathiolation of cysteines and their number increases with more intensive NO stimulation. NO in this reaction binds to O_2^- and forms low levels of intracellular $ONOO^-$, which glutathiolates by glutathione (GSH) the reactive cysteine thiols. Intracellular Ca^{2+} decreases (272). Thus, by removing O_2^- and preventing the formation of $OONO^-$, O_2^- scavengers actually blocked NO-induced vasodilation. High concentrations of $OONO^-$ caused irreversible oxidation of key thiols and prevented NO-induced vasodilation. An example is NAD(P)H oxidase in phagocytic cells that produces large amount of O_2^-, whereas the NAD(P)H oxidase in non-phagocytic cells produce small amounts of O_2^- that facilitates their signaling role (273).

Estrogen and NO in the vasculature

There are several reports that E2 can modulate **eNOS** activity and mRNA expression. E2 replacement increased the eNOS in OVX rat kidneys (274). An increase in eNOS mRNA has been observed in the aorta of pregnant or E2-substituted ovariectomized (OVX) rats

(275). E2 upregulated calcium-dependent eNOS activity and mRNA in guinea pig non-vascular tissues (204). E2 induced a rapid calcium-dependent increase of eNOS in bovine aortic EC that was mediated by estrogen receptor suggesting non-genomic mechanism (276).

In animal studies, the acute E2 administration to OVX ewes selectively increased the endothelium-derived eNOS activity, while daily E2 increased both eNOS from endothelium and nNOS derived from smooth muscle (206). E2 treatment of OVX ewes induced the eNOS protein expression primarily in the endothelium of uterine arteries (207). In studies on cultured ECs, the physiological dose of E2 in ovine fetal pulmonary artery EC rapidly, in 5 min, doubled the eNOS activity, but there were no changes in protein or mRNA expression (113). Ethinylestradiol (EE2) in Arnal's study on cultured bovine aortic EC after long-term treatment (24-48 hours) did not induce any change in eNOS activity, protein or mRNA expression, however EE2 in dose- and time- dependent manner decreased O_2^- anion production and thus prevented bioactive NO degradation (277). In permanent human endothelial hybrid cell line EA.hy 926 (140), EE2 did enhance eNOS mRNA and protein expression as a result of increased eNOS promoter activity mediated by an increased activity of transcription factor Sp1 (278).

nNOS can also be modulated by estrogens. Pregnancy induced nNOS activity in several tissues in the rat (279;280) and E2 increased nNOS mRNA in neural tissue of OVX rats (281). E2 treatment increased nNOS expression in the hypothalamic paraventricular nucleus of OVX rats and these effects were ERβ dependent (282). Increased nNOS activity and mRNA induced by E2 was shown in guinea pig cerebellum as well as in nonneuronal tissues (204).

The effect of E2 on abundant NO production by **iNOS** was also addressed. Uterine arteries from follicular-phase sheep expressed significantly more eNOS after LPS exposure than did uterine arteries from luteal-phase ewes. In contrast, iNOS was not detected in these arteries after LPS exposure. This suggests the role of E2 in regulating uterine artery responses to LPS (283). E2 significantly attenuated the excessive NO production in LPS-induced OVX rats (284) suggesting that E2 may be beneficial in pathological situations associated with intense NO production. E2 demonstrated its modulatory effects in cytokine (TNFα)-induced iNOS activity in rat VSM where it decreased iNOS mRNA and protein expression (285).

Serine/threonine kinase AKT as a mediator of E2 action on NO production

An increase in NO production by eNOS seems to be a common factor for beneficial effects of E2. We (138) and others (136;137) have shown that E2 can act through PI(3)K to phosphorylate the serine threonine kinase AKT/PKB. This results in the phosphorylation on serine 1177 of human eNOS and increased NO production. Since AKT can activate eNOS and E2 activates AKT, this pathway could explain the E2 induced increase in NO (138). We showed that in bovine adrenal medulla microvascular endothelial cells (BAMEC) the phosphorylation of AKT occurred rapidly within 1 min, while no further changes occurred between 30 and 60 min. Treatment of HUVEC with increasing concentrations of E2 resulted in maximum phosphorylation of AKT (Ser473) as well as phosphorylation of eNOS (Ser1177) with E2 10^{-8} M, which corresponds to physiological levels of serum E2. The PI(3)K inhibitor wortmannin (W) and the ER

specific antagonist ICI 182,780 blocked this increase. We assessed the functional significance of E2-induced increase in NO by the isometric tension assay on OVX rat aortic rings. We found that Ach-induced dilation of aortic rings from OVX rats was more intensive and started at lower Ach concentrations in E2 treated rings compared to placebo. The PI(3)K inhibitor W blocked this effect that confirmed the involvement of the PI(3)K pathway. We observed the qualitatively similar effect in OVX rat cerebral microvessels. E2 treatment dilated pial microvessels of OVX rats more compared to controls. Tamoxifen resulted in vasodilation that was greater than observed with E2 and there was no further flow- or Ach-mediated vasodilation. Addition of W caused profound vasoconstriction. However, there was still present a vasodilatory response to nitroprusside, an endothelium independent vasodilator, and vessels dilated even when Ca^{2+} was removed.

Evidence that these E2 effects are through a cell membrane receptor was provided by Haynes and Hisamoto (135;137). They used membrane-impermeable conjugate of E2, E2-BSA that increased eNOS activity in EC similarly to non-conjugate E2 which suggested that there was a membrane associated form of the receptor. The transcriptional inhibitor actinomycin D did not block PI(3)K activity (136) nor phosphorylation of AKT (137) after stimulation of EC with E2, which was consistent with a rapid non-nuclear effect of ER stimulation on eNOS activation. In contrast, ICI 182,780 efficiently inhibited the phosphorylation of eNOS and NO release (135;138), PI(3)K activation (136) and phosphorylation of AKT (137) after E2 stimulation in ECs.

The role of calcium in eNOS activation by estrogen

The precise activating molecular mechanism of NO release from EC after E2 treatment in relation to dependence of calcium (Ca^{2+}), is still not known. eNOS is a Ca^{2+}/CaM – dependent enzyme and its activation by agonists such as Ach require an increase in intracellular Ca^{2+} (286). It was shown that E2 acutely induced translocation of eNOS by a Ca^{2+}–dependent and estrogen receptor-mediated mechanism (93;114;276). An interesting study on the acute effects of E2 on eNOS activity induction in human airway epithelial cells also showed a dependence on Ca^{2+} (287).

However, recent evidence supports that in EC several agonists such as fluid shear stress (288), insulin (289) and estrogen (163) can activate eNOS and enhance NO production in the absence of Ca^{2+}. Thus, studies on nongenomic rapid actions of E2 on ECs in relation to the Ca^{2+} mobilization differ in their findings. The study of Caulin-Glaser showed for the first time that treatment with E2 could rapidly induce NO release from human EC without requiring increases in cytosolic Ca^{2+} (163).

The role of calcium in the nongenomic activation of PI(3)K/AKT pathway is also not yet established. eNOS phosphorylation and subsequent NO release in EC has been shown to be mediated by PI(3)K/AKT pathway at resting Ca^{2+} concentrations (134;139;290). In agreement, Haynes et al. showed that E2 rapidly activated and phosphorylated eNOS through PI(3)K/AKT pathway by Ca^{2+}-independent manner, thus E2-stimulated endothelial NO release did not require a rise in free intracellular Ca^{2+} (134;135). Hisamoto evaluated the role of extracellular and intracellular Ca^{2+} on E2 activation of PI(3)K/AKT in transformed rat lung endothelial cells (TRLEC). Elimination of

extracellular Ca^{2+} by EGTA for 1 min inhibited the E2-induced AKT and eNOS activation. Elimination of intracellular Ca^{2+} by BAPTA-AM, an intracellular Ca^{2+} chelator, completely abolished the E2-induced AKT and eNOS. Moreover, elimination of both, intra- and extracellular Ca^{2+} with EGTA for 15 min, also abolished the PI(3)K/AKT activation. Thus, these results suggest that Ca^{2+} mobilization is required for the E2-induced AKT and eNOS activation through PI(3)K/AKT pathway (137).

I.6. NAD(P)H Oxidases

The structure and function of the NAD(P)H oxidases

Reactive oxygen species (ROS) participate in the pathophysiological vascular response that leads to hypertension (291;292).

Oxygen is necessary for cellular respiration and cells have developed several enzyme systems that use oxygen as an acceptor of electron transfer (293). The NAD(P)H oxidase is one of the enzyme systems that contribute in oxygen reduction (294). In VSMC and EC, the NAD(P)H oxidase is the predominant superoxide-producing enzyme. Furthermore, the activity of vascular NAD(P)H oxidase is regulated by cytokines (295), hormones (296;297) and mechanical forces (298).

The NAD(P)H oxidases were well characterized initially in neutrophils where two membrane components, $p22^{phox}$ and $gp91^{phox}$, comprise the cytochrome *b*558. Other important components include the cytoplasmic subunits $p47^{phox}$, $p40^{phox}$, $p67^{phox}$ and the small GTP-binding protein Rac. In unstimulated phagocytes the oxidase is unassambled and inactive. When phagocytic cells are activated, the cytosolic subunits translocate to cytochrome *b*558 at the membrane, which results in the activation of the oxidase and the well characterized oxidative burst (299). A deficiency of one of the four *PHOX* subunits that comprise the NAD(P)H oxidase causes chronic granulomatous disease (CGD) (300;301). Accordingly, phagocytes from patients with CGD fail to generate a respiratory burst and produce superoxide (O_2^-) (302;303). An important initiating event in the activation of the NAD(P)H oxidase is the phosphorylation of $p47^{phox}$ (304;305). The crystal structure of $p47^{phox}$ reveals that intermolecular interaction between two tandem

Src homology domains auto-inhibits $p47^{phox}$ binding to $p22^{phox}$ (306). Phosphorylation of $p47^{phox}$ causes a loss of this auto-inhibitory interaction, which allows binding of $p47^{phox}$ and $p22^{phox}$ and initiates the translocation of cytoplasmic components to the membrane that leads to the respiratory burst (306).

Components of the neutrophil NAD(P)H oxidase have also been identified in cells other than phagocytes (307). Non-phagocytic NAD(P)H oxidase was also found in the carotid bodies of the carotid sinus (308), human glomerulal mesangial cells (309), vascular smooth muscle cells (VSMCs) of the rat thoracic aorta (296;310) and of the calf pulmonary artery (311), bovine coronary artery endothelial cells (ECs) (312), human umbilical vein ECs (313) and human and rabbit aortic adventitia fibroblasts (314;315).

In ECs, all of the NAD(P)H oxidase subunits characterized in phagocytes, including $gp91^{phox}$, are expressed (316). Using $gp91^{phox}$ knockout mice, it was shown that $gp91^{phox}$ was required for phorbol myristate acetate (PMA) stimulated, but not basal endothelial NAD(P)H oxidase activity (317). The functional relevance of $gp91^{phox}$ expression in ECs was assessed in the same study. PMA induced reactive oxygen species (ROS) secretion in ECs and in endothelium-intact aortic segments, but not in endothelium-denuded aortic segments (317). Furthermore, NAD(P)H oxidase inhibitors such as apocynin or diphenyleneiodonium (DPI) were shown to reduce ROS production (313;318-320), thus providing confirmatory evidence that the source of the ROS was an NAD(P)H oxidase. The activity of the NAD(P)H oxidase in ECs was also confirmed in perturbed ECs and linked to atherogenic LDL (319). Induction of HUVEC by oxLDL caused transient induction of $gp91^{phox}$ mRNA expression (321). NAD(P)H oxidase-dependent endothelial O_2^- formation is constitutive, continuous and on a much lower level compared to the

oxidative burst produced by induced phagocytes (322). This lower NAD(P)H oxidase activity seems to be mediated by the ~100-fold lower expression of subunit $gp91^{phox}$ in ECs compared with granulocytes (321).

In the past few years, a family of $gp91^{phox}$-like proteins was cloned. They were termed the non-phagocytic NAD(P)H oxidase (NOX) proteins. NOX1 is a homolog of $gp91^{phox}$ (323), NOX2 is also known as $gp91^{phox}$ (324). NOX1, NOX2, as well as NOX3, NOX4 and NOX5 (325) likely account for some of the reactive oxygen generation in non-phagocytic cells of different tissues. NOX2 (313) and its homologue NOX4 (326) were found in ECs, where they function as an oxygen sensor (327). It also was found that the expression of NOX4 in ECs markedly exceeded that of other NOX proteins, including $gp91^{phox}$/NOX2 (328). Indeed, a high expression of NOX2 and NOX4 mRNA in HUVEC was confirmed in the research of others. Interestingly, NOX4 was expressed at ~100-fold higher levels compared with NOX2. They concluded that differential mRNA expression of NOX family members and their regulatory proteins $p47^{phox}$ and $p67^{phox}$ could be involved in the control of ECs spreading, motility and cell-cell adhesion (329).

NAD(P)H and Estrogen

Estrogen (E2) is considered to be a key element in gender-specific cardioprotective effects. E2 lowers circulating lipids and improves lipoprotein profiles (3;18), and it also increases endothelium-dependent vasodilatation (152). A significant E2-stimulated increase in the bioavailable NO is also known (85). The antiatherogenic properties of NO are well documented (330). Therefore, it has been proposed that the positive

cardioprotective effects of E2 are based on an increased production of NO in the vascular endothelium (19;160;331;332). However, an increase in bioavailable NO can occur even without an increase in NOS activity and NO production, by a decrease in O_2^- production (277;333). This occurs because O_2^- combines with NO and removes it by forming peroxynitrite, a potent oxidant which has further negative vascular effects (221). Enhanced O_2^- production reduces the bioavailability of NO in CVDs including atherosclerosis. It also promotes the NO-sensitive expression of proatherosclerotic gene products such as adhesion molecules or chemokines in ECs (71;334). NAD(P)H oxidase is an important source of O_2^- in HUVEC (273;319). It was demonstrated that the activity of this enzyme is a critical factor in the early phase of atherosclerosis (335). One group of investigators showed that at physiological concentrations, E2 caused in HUVEC a time- and concentration-dependent decrease in the expression of the NAD(P)H oxidase subunit $gp91^{phox}$ at both mRNA and protein level. Furthermore, O_2^- formation stimulated by phorbol-ester was significantly attenuated in E2 treated cells and this effect was mimicked by blocking $gp91^{phox}$ protein synthesis with an appropriate antisense oligonucleotide. Since the effect of E2 on $gp91^{phox}$ was reversed by an ER antagonist, the authors suggested that these potentially atheroprotective effects of E2 were mediated by the estrogen receptors (336). In addition, other investigators found that E2 attenuated the NAD(P)H subunit $gp91^{phox}$ in HUVEC that were induced by angiotensin-II (AII), but the protein expression of NAD(P)H oxidase was not altered by ER inhibition. On the other hand, E2 was able to inhibit the expression of AII type 1 receptor (AT_1R), which could explain how E2 diminished the oxidative stress in ECs (337). Also Laufs et al. showed that treatment with E2 completely prevented AII-induced NAD(P)H oxidase subunit Rac-

1 GTPase activity and ROS production in VSMCs. In their study, E2 decreased AII-induced and basal Rac-1 mRNA and protein expression as well as Rac-1 activity in a time and concentration dependent manner (338). Our own findings (see *Chapter II* and *Appendix 1*) showed only little variation in the expression of components of NAD(P)H oxidase with ovariectomy and E2 replacement, but E2 reduced migration of the $p47^{phox}$ regulatory component to the membrane and lowered the production of O_2^- by NAD(P)H oxidase (339). Sumi et al. also found that E2 decreased $p47^{phox}$ in human monocytic THP-1 cells via decreased NFκB activation (340). In contrast to the studies of Arenas et al. who found a modulation of vascular function in E2-depleted rats (341), we did not find that reducing O_2^- with radical scavengers or inhibitors of production had any effect on vascular function. However, the decrease of ROS still might have an impact on intracellular signalling and on the expression of inflammatory molecules.

I.7. Apoptosis

Caspases

The majority of apoptosis-inducing agents effect through the activation of caspases, notably caspase-3, an ultimate apoptosis executioner (342).

Caspases (cysteine aspartases) are highly specialized proteases that lead to apoptosis. Caspases are activated by divergent pro-apopotic stimuli (343-346). To date, 14 caspases have been identified in mammals (345). Caspases have distinct roles in apoptosis and inflammation. Caspases -2, -3, -6, -7, -8, -9, and -10 are thought to be centrally involved in cell death. The primary role of caspases -1, -4, -5 and -11 seems to be in cytokine processing. In apoptosis, caspases are directly responsible for proteolytic cleavages that lead to cell disassembly (effector caspases). Initiator caspases are involved in upstream regulatory events of the caspase cascade. The observation that distinct death signals result in the same manifestations of apoptosis is explained by the finding that effector caspases are activated by different initiator caspases, each of which is activated by a set of proapoptotic signals (345).

Apoptosis is mediated by two central pathways: the extrinsic (or death receptor) pathway and the intrinsic (or mitochondrial) pathway (347). Human caspases are subdivided into upstream (apical, signaling) caspases (caspases 2, 8, 9, 10 and 12) and downstream (effector, executioner) caspases (caspases 3, 6 and 7) (348). Extrinsic signaling is initiated at the apex by the binding of a death ligand trimer such as Fas associated protein with the death domain (FADD) or with a soluble extracellular protein (eg. TNFα)

(349;350). FADD recruits procaspase-8 into the death-inducing signaling complex (DISC) (351;352). This results in the activation of caspase-8 that cleaves procaspase-3 and causes proteolysis of cellular substrates (353). Unlike the extrinsic pathway that mediates a specialized set of death stimuli, the intrinsic (mitochondrial) apoptosis pathway is activated by various extracellular and intracellular stimuli. Extracellular stimuli include deficiencies in growth factors, radiation and other chemical and physical stresses (354), whereas intracellular stimuli include oxidative stress, DNA damage and protein misfolding (355-357).

Mitochondria and Bcl-proteins

Mitochondria play a key role in the regulation of apoptosis (358;359). Mitochondrial intermembrane space contains several proteins that are released through the outer membrane and that are subsequently involved in the destruction phase of apoptosis (360-362). Mitochondrial dysfunction could be caused by factors such as mitochondrial membrane potential ($\Delta\Psi_m$) collapses (363), indicating the opening of a large conductance channel known as the mitochondrial permeability transition pore (PTP) (364). The PTP is a large conductance pore that evolves in mitochondria following necrotic or apoptotic signals. The PTP is permeable to solutes with a molecular mass of ~ 1500 daltons. The PTP comprises the inner and outer mitochondrial membrane proteins, such as the adenine nucleotide translocator (ANT) or porin, a voltage-dependent anion channel (VDAC). Opening of the PTP results in mitochondrial depolarization that leads to the swelling of the mitochondria (358;365).

Bcl-2 was discovered as an oncoprotein that supports neoplastic growth not by stimulating cellular proliferation, but rather by blocking cell death (366). Recently, a number of proteins functionally and structurally related to Bcl-2 have been discovered. The Bcl-2 family members play a pivotal role in deciding whether the cell will survive or undergo apoptosis. It includes both pro- as well as anti-apoptotic molecules. Bcl-2 family members possess up to four conserved Bcl-2 homology (BH) domains designated BH1, BH2, BH3 and BH4, which correspond to α-helical segments (359). Many of the antiapoptotic members display sequence conservation in all four domains, e.g. Bcl-2 and Bcl-xL that promote cell survival (367). The pro-apoptotic molecules frequently display less sequence conservation of the first α-helical segment, BH4. Bcl-2 members that display sequence homology only within the BH3 domain, so-called "BH3-domain only", are all pro-apoptotic, e.g. Bid, Bad, Bim. Bcl-2 is a multifunctional protein. Bcl-2 and some of its anti-apoptotic homologues such as Bcl-xL are capable of forming homo-as well as heterodimers and they also have an ability to become integral membrane proteins (368). The Bcl-2 family members are located in the cytoplasm and at intracellular membranes, including the outer mitochondrial membrane. A change in the balance between prosurvival and prodeath Bcl-2 family members results in the translocation of pro-apoptotic Bcl-2 family members from the cytoplasm to the mitochondrial outer membrane and this initiates a cell death program involving decreases in mitochondrial membrane potential, opening the PTP, decreases in nucleotide exchange and the release of cytochrome-c (369), Smac/Diablo (370) and apoptosis-inducing factor (AIF) (371) Increases in the concentration of cytochrome-c result in the nucleation of a multiprotein complex known as the apoptosome, which is comprised of the apoptosis protease-

activating factor-1 (Apaf-1) adapter protein, cytochrome-c, dATP and the protease caspase-9. This complex then activates effector caspase-3, indispensable for apoptosis.

In the presence of survival factors, the activity of prosurvival Bcl-2 family members prevails over the activity of prodeath Bcl-2 family members. The PI(3)K and its downstream target, the serine-threonine kinase AKT, were found to be required for the growth factor-dependent survival of a wide variety of cultured cell types (372;373) and were also found to be implicated in the suppression of apoptosis (374). The first component of the apoptotic machinery to be phosphorylated by PI(3)K/AKT was the Bcl-2 family member Bad. Bad function is modulated by phosphorylation at two sites, serine 112 (Ser-112) and serine 136 (Ser-136) (375;376). In the absence of phosphorylation at these sites, Bad is thought to induce cell death, possibly via the formation of heterodimers with Bcl-xL. Phosphorylation has been correlated with the binding of BAD to the 14-3-3 protein, which may sequester Bad from Bcl-xL, thus promoting cell survival. For instance, it was shown that IGF-1 through PI(3)K/AKT phosphorylated BAD and promoted cell survival (377;378). This suggests that through an effect of growth factors, AKT can phosphorylate Bad at Ser-112 and Ser-136 in vivo, which leads to the inactivation of Bad and promotes survival.

Apoptosis and Estrogen

Development of atherosclerotic plaques in the vascular endothelium occurs in lesion-prone regions that are characterized by increased endothelial cell turnover rates (379). The enhanced EC turnover is most likely secondary to an increase of programmed cell death or apoptosis. The first report that apoptosis can be induced in EC was published in

1991 by Robaye and coworkers (380). They showed that the inflammatory cytokine TNFα caused massive death of bovine EC in primary culture in a concentration- and time-dependent manner, which was accompanied by massive DNA fragmentation quantitatively correlating with a proportion of apoptotic cells. Another group of investigators showed that apoptosis of EC was suppressed by NO via inhibition of effector caspases (381). High glucose concentrations, a situation similar to that in diabetes, triggered EC apoptosis (382). Furthermore, oxidized low-density lipoprotein (oxLDL), which plays a key role in atherogenesis, as well as reactive oxygen species (383) and angiotensin-II (384), stimulated apoptosis in EC (385). On the other hand, it was shown that endogenous anti-atherosclerotic factors could prevent apoptosis. For instance, shear stress has been shown to completely prevent apoptosis induced by various stimuli in primary endothelial cells (386-388) and in osteoblasts (389). In addition, other factors such as antioxidants have been shown to prevent endothelial cell apoptosis induced by TNFα (390), oxLDL (385;391) and endotoxin (392). Morover, growth factors such as IGF-1 (377) and the female hormone estrogen were also found to inhibit apoptosis in human EC (135;393;394).

The mechanism of antiapoptic E2-actions was established in many cell types, particularly in cancer cell lines. E2 is a well-characterized mitogen for mammary tissues and epithelial cells of the female reproductive tract. E2 acted as a survival factor for breast cancer probably through the prevention of programmed cell death, apoptosis (395). E2 showed antiapoptotic influence in neurons (396) and epithelial cells of the mammary gland (397), and E2 also affected murine osteoclasts by promoting apoptosis and limiting their lifespan, thus preventing the excessive bone loss before and after menopause (398).

Furthermore, in some ER-negative cells, such as CHO cells, the overexpression of ER led to apoptosis (399) probably through activation of $p38^{MAPK}$ (400). On the other hand, E2 promoted cell survival in ER-positive MCF7 cells (401;402) and this effect was mediated by PI(3)K/AKT (133). The mechanism of E2 antiapoptotic actions in many cell types was explained by the fact that E2 induced transcription of the Bcl-2 gene and increased expression of Bcl-2 protein (397;401;403). Razandi and al. showed that E2 prevented chemotherapy or radiation-induced apoptosis of ER-positive breast cancer cells MCF-7 through the plasma membrane ER (128). A group of investigators examined the signaling mechanism of E2 with respect to the regulation of cell survival in MCF7 breast cancer cells. They showed that rapid treatment with E2 significantly reduced apoptosis induced by TNFα, H_2O_2 and serum withdrawal by using signal transduction pathways that led to phosphorylation and inactivation of proapoptotic protein BAD. Furthermore, the mechanism by which E2 prevented apoptosis was nongenomic (404). Based on these studies, the actions of the membrane ER in breast cancer could underlie the ability of E2 to effect cell survival *in vivo* (405).

I.8. Angiogenic Effects of E2

Angiogenesis

Angiogenesis is the process by which blood vessels are assembled to form functional vasculature. Embryonic vessels are assembled from endothelial precursors called angioblasts [2] (406) in the de novo process of vasculogenesis in the absence of preexisting vascular structures. In angiogenesis, new vessels arise by budding and sprouting from larger, existing vesels (407). In vasculogenesis, mobilization of endothelial precursors requires the expression of growth factors, in particular basic fibroblast growth factor (bFGF) and vascular EC growth factors (VEGFs) (407;408). Four alternatively spliced isoforms of vascular endothelium-specific growth factor (VEGF), VEGF-A, VEGF-B, VEGF-C and VEGF-D, members of the platelet-derived growth factor family, interact with specific tyrosine kinase receptors (409). These include Fms-like tyrosine kinase-1 or VEGF receptor-1 (flt-1)(VEGFR-1) for VEGF-A, fetal liver kinase/kinase-insert domain receptor or VEGF receptor-2 (flk-1/KDR)(VEGFR-2) for both VEGF-A and VEGF-C; and a tyrosine kinase receptor flt-4 or VEGFR-3, which interacts mainly with VEGF-C. All VEGFs stimulate receptor autophosphorylation (34). VEGF initiates angiogenesis by stimulating extracellular matrix (ECM) degradation as well as migration, proliferation and differentiation of ECs into newly formed capillaries (410). VEGF induces molecules such as the matrix metalloproteinase-1 in HUVEC (411) and VSMC (412) which is important for the cellular migration processes. VEGF also increases the expression of urokinase type plasminogen activator (uPA) (413), urokinase type plasminogen activator

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[2] vascular endothelial cells that have not yet formed a lumen

receptor (uPAR) (414) and plasminogen activator inhibitor-1 (PAI-1) (415) which are involved in extracellular proteolysis and thus mediate angiogenesis (416;417). The loss of VEGF-receptor (VEGFRs) gene results in embryonic death between days 8.5 and 9.5, indicating the importance of VEGFRs in vasculogenesis and angiogenesis (418;419). Furthermore, vasculogenesis originating from ECs precursors requires activation of VEGFR-1 on the surface of newly differentiated cells (419;420).

VEGF exhibit two major biological activities: one is the capacity to stimulate vascular EC proliferation (421;422) and the other is the ability to increase vascular permeability (423;424), which allows large proteins and cells to cross the endothelial lining of vessels. VEGF also promotes the migration of bovine (425) and human (426) EC and elicits DNA synthesis in HUVEC (426). VEGF induces the survival of EC through the VEGFR-2 and PI(3)K/AKT signal transduction pathway (427;428). Expression of VEGF and VEGFR-2 are increased by inflammatory cytokines such as IL-1α, IL-6 and TNFα (429;430). VEGFR-2 mRNA is increased in TNFα-stimulated HUVEC (431).

Angiopoietins / Tie-2

The angiopoietins represent a newer class of angiogenic factors that like VEGF are largely specific for the vascular endothelium (432-436). Angiopoietins are three structurally related proteins, Angiopoietin-1 (Ang-1), Angiopoietin-2 (Ang-2) and Angiopoietin-3/4 (Ang-3 is the murine orthologue of human Ang-4) (409;432;435;437).

Ang-1 has an essential role in vascular remodelling during development. The phenotype of mice lacking Ang-1 is very similar to the Tie-2 knockouts. Embryos homozygous for the disrupted Ang-1 gene died by embryonic day 12.5 (438). Embryos display retarded

heart growth. In addition, the primary capillary plexus fails to differentiate, whereas the existing vessels appear dilated and display low connection of ECs with the extracellular matrix and pericytes (420;438). Ang-1 is a primary physiologic ligand for Tie-2 and it has critical in vivo angiogenic actions since it mediates reciprocal interactions between the endothelium and the surrounding matrix and mesenchyme. Ang-2 knockouts generally die within two weeks of birth. They have normal embryonic vascular development but manifest defects in vascular remodelling postnataly. Mice lacking Ang-2 also exhibit major lymphatic vessel defects, which can be genetically rescued with Ang-1. However, this repair does not correct the defects in angiogenesis (439). Thus Ang-2 acts as a Tie-2 agonist in lymphangiogenesis but as an antagonist in angiogenesis (439;440).

The action of angiopoietins occurs through endothelial cell-specific receptor tyrosine kinases (RTK) that has two members, Tie-1 and Tie-2 (or Tek, tunica interna EC kinase) (441-443). Whereas Tie-2 is a well-known receptor for angiopoietins (Angs), ligands for Tie-1 have yet to be identified. Tie-1 is currently an orphan receptor but may dimerize with Tie-2 and modulate Tie-2 signaling (444). Tie-receptors are essential for developmental vascularization and have role in promoting microvessel maturation and stability (436). Targeted disruption of the *Tie-1* gene in mice results in a lethal phenotype characterized by extensive hemorrhage and defective microvessel integrity (420;445). Transgenic mice deficient in *Tie-2* have defects in vessel sprouting and remodelling and die at around mid-gestation (420). Embryos deficient in Tie-1 demonstrate leaky syndrome manifested by impaired endothelial integrity that results in oedema and

subsequently localized haemorrhage. All neonates homozygous for *tie-1* mutation die immediately after birth due to failure to expand alveoli in their lungs (420).

Angs act as endothelial survival factors. Ang-1 is normally present at low levels throughout adult tissues and provides a stabilizing pro-survival signal. For example, Ang-1 reduces vascular permeability produced by VEGF (446). This action of Ang-1 likely reduces microvascular leakage that occurs in chronic inflammatory diseases including diabetic rethinopathy and nephropathy (410). Ang-1 also promotes antiapoptotic pathways (447;448). On the other hand, binding of Ang-2 to Tie-2 generally inhibits its activity, which allows VEGF to predominate and initiate angiogenesis (436). Ang-2 also has a pro-survival effect and can activate the AKT survival pathway when expressed at high levels around sprouting vessels (449). Vascular quiescence is maintained by constitutive Ang-1/Tie-2 signaling with an Ang-1:Ang-2 ratio in favor of Ang-1. Following EC activation, Ang-2 is rapidly released from Weibel-Palade bodies and transcriptionally upregulated in EC. Ratio of Ang-1:Ang-2 moves in favor of Ang-2 which destabilizes the endothelium that becomes more responsive to other cytokines, such as TNFα (450).

Signaling of the Ang-1/Tie-2 receptor pathway

Signaling of the Ang-1/Tie-2 pathway has recently been intensively investigated. It was demonstrated that the p85 regulatory subunit of PI(3)K associates with a binding motif of tyrosine 1101 on the Tie-2 molecule in a phosphotyrosine-dependent way via its carboxyl SH2 domain. This association correlates with the activation of PI(3)K and its downstream target AKT (451;452). AKT1 is a key molecule for angiogenesis (453;454). Activation of

AKT leads to attenuation of apoptosis and activation of eNOS (447;448;455-457). In addition to survival, PI(3)K pathway also links Angs to endothelial migration. It was shown that PI(3)K inhibitors reduced the Ang-1 stimulated migration of HUVEC (458). Although Ang-1 has been shown to promote survival of ECs via the PI(3)K/AKT pathway (456;457), the relevant AKT substrates that might mediate its effects on ECs were determined by Daly and co-workers. They demonstrated that Ang-1, via AKT activation, is a potent inhibitor of the forkhead transcription factor FKHR (FOXO1). At the same time they identified a nuclear signalling pathway through which Ang-1 inhibited FKHR-mediated changes in gene expression, including the Ang-1 antagonist Ang-2, and FKHR-induced apoptosis. Thus, Ang-1 was shown to be an important regulator of EC survival and blood vessel stability (459). Unexpectedly, in a later study, the same group of investigators showed that Ang-2 also functioned as a Tie-2 agonist and supported AKT activity, which provided negative feedback on FOXO1-regulated transcription. In addition, autocrine Ang-2, like Ang-1, activated Tie-2/AKT signalling *in vivo*. Ang-2 subsequently inhibited vascular leak (460).

Separately from the p85 regulatory subunit of PI(3)K, other molecules also associate with Tie-2. Upon ligation of Ang-1, Tie-2 receptors dimerize, autophosphorylate specific tyrosines and recruit adaptor protein Grb2 and tyrosine phosphatase SHPTP2 (461). Finally, a novel docking molecule, Dok-R, was shown to bind Tie-2 in a phosphotyrosine-dependent manner (462). Ang-1 was also reported to activate Erk1/Erk2 and p38 members of the mitogen-activated protein kinases in ECs (448).

Estrogen and Angiogenic Factors

It is known that steroidal hormones play a role in the regulation of angiogenesis (463). E2 has an important role in angiogenesis within the uterine endometrium during each menstrual cycle (464). The monthly cycling of E2 production (465) and its relationship with the timing of vessel formation suggests a critical role for E2 in vascularization (466). In ovariectomized baboons, acute administration of E2 rapidly increases endometrial VEGF mRNA expression (467). E2 has been shown to regulate expression and secretion of VEGF by human glandular epithelial cells of the endometrium and to promote tube formation in co-cultured microvascular ECs (468), thus regulating endometrial angiogenesis. E2 also can upregulate VEGFR-2 in human microvascular ECs by acting through ERα (469).

Expression patterns in ovarian tissue suggest that steroidal hormones influence the regulation of expression of the Angs. Ovarian follicle development depends on sequential regulation of vascular outgrowth and vascular regression. It has been reported that in ovarian tissue in the presence of abundant VEGF, Ang-2 can promote vessel sprouting by blocking a constitutive Ang-1 signal, whereas in the absence of VEGF the Ang-2 inhibitory effect on Ang-1 can contribute to vessel regression. Thus, the Ang ratio of the activation state of the Tie-2 receptor may regulate the responsivness of EC to VEGF (435). Consistent with its role in initiating angiogenesis, the expression of Ang-2 appears to be more regulated than that of Ang-1. We also found in non-reproductive tissues of OVX rats that E2 increases the expression of Ang-2 and Tie-2, whereas E2 decreases the expression of Ang-1 (470). This result is consistent with the pro-angiogenic activity of E2 because an increase in the ratio of Ang-2 to Ang-1 decreases the inhibitory effect of Ang-

1 (435). This effect is associated with decreased phosphorylation of Tie-2, which indicates decreased receptor activity (470).

Migration of ECs or capillary formation in response to angiogenic mediators such as bFGF or Matrigel, an extract of the basement membrane, is also enhanced by E2 (192). The angiogenic activity induced by bFGF is markedly decreased in OVX mice, but it is restored when animals are continuously repleted from E2-implants (192).

NO Plays a Role in E2-mediated Angiogenesis

Numerous studies have shown that E2 can regulate NOS and its product NO (182). E2 upregulates eNOS in human aortic endothelial cells (471). Disruption of ERs in male patients has been associated with endothelial dysfunction (188). E2 regulates uterine NOS expression (472). Short-term administration of E2 augments NO-mediated vasodilation in the human forearm (473) and ovine uterine arteries (474) by mechanisms that may involve increased basal NO release (163;475) and increased eNOS activity (113;114).

The role of NO in angiogenesis has been questioned. Chemically derived NO inhibited serum-induced migration of HUVEC (476). L-NMMA, an eNOS inhibitor, increased vascular density in the chick embryo chorioallantoic membrane (477). *In vitro*, exogenous NO but also a cell permeable cGMP analogue reduced tube formation in the matrigel tube formation assay (478). More recent studies showed the importance of NO in the angiogenic response to VEGF. Indeed, NO mediated the VEGF-induced proliferation in HUVEC and stimulated network structures in an NO-dependent manner.

Furthermore, exposure of HUVEC to VEGF increased NO production mediated by activation of tyrosine and PI(3)-kinases (479). Supporting these data, another study showed that endogenous endothelium-derived NO maintained the functional expression of integrin, a mediator for endothelial migration, survival and angiogenesis (480). In the mouse model with targeted disruption of eNOS gene, investigators showed that vascular injury caused an increase in neointimal proliferation. This finding was consistent with the protective role of eNOS in preventing vascular intimal formation *in vivo*. Furthermore, pregnancy suppressed the response to vessel injury in both wild-type and eNOS mutant mice suggesting that this effect was due to circulating estrogens (481).

E2 can modulate adhesion molecule expression

E2-mediated alteration in the expression of certain adhesion molecules has been shown in endothelial cells (71;482). E2 was found to have strong inhibitable effects on the cytokine IL-1 mediated expression of E-selectin and VCAM-1 (71). This likely occurs through E2 mediated activation of eNOS and increased NO production (135;136;138;163). E2 also inhibits leukocyte adhesion by blocking the secretion but not the transciption of interleukin-8 (IL-8) and monocyte chemotactic protein-1 (MCP-1) (483). These studies support the role of E2 as a promoter of a "healthy vascular wall" and a quiescent endothelium. In contrast, Cid and co-workers found that E2 produced a dose-dependent increase in attachment of polymorphonuclear leukocytes (PMN) to ECs in culture in response to TNFα. They showed that E2 increased TNFα-induced adhesivness of HUVEC for leukocytes via increased expression of the endothelial adhesion molecules E-

selectin, intercellular adhesion molecule type-1 (ICAM-1) and vascular cell adhesion molecule type-1 (VCAM-1). E2 treatment resulted in increased levels of mRNA for these cytokines at early time-points, whereas at later time-points the expression of mRNA for these adhesion molecules was reduced in the presence of E2 (482). This mechanism could play a role in the development and perpetuation of chronic inflammatory diseases in females, especially before menopause.

I.9. Rational and Objectives of the Research

Atherosclerosis is a major cause of CVDs in postmenopausal women. Cardiovascular disease (CVD) occurs less often in pre-menopausal women than in same-aged men (19;484). Population studies show that postmenopausal women on hormone replacement therapy (HRT) with estrogen (E2) have less CVD than women not taking them (13). However, recent prospective primary and secondary prevention trials failed to demonstrate an overall protective effect from HRT (6;7;10;16).

ROS and Atherosclerosis

E2 is known to increase the bioavailability of NO (85). The ability of E2 to increase NO is ER-dependent but independent of changes in gene expression, resulting in the activation of eNOS (114;135). On the other hand, E2 has been shown to up-regulate eNOS mRNA expression in animal pulmonary and aortic ECs (485;486). Since NO is a well-known antiatherogenic agent (330), it has been proposed that the beneficial effects of E2 on the vasculature are mediated by an increased production of NO by the endothelium (487). An increase in bioavailable NO can occur even without an increase in NOS activity and NO production, through a decrease in O_2^- production (277;333). This occurs because O_2^- combines with NO to form peroxynitrite, a potent oxidant, which has further negative effects on the endothelium (221). Enhanced O_2^- formation reduces the bioavailability of NO in CVDs and atherosclerosis (488). NAD(P)H oxidase is an important source of O_2^- in human ECs (273;319) and an increasing body of evidence

demonstrates that the activity of this enzyme plays a critical role in the early phase of atheroslerosis (335).

Thus, we investigated the hypothesis that E2 treatment of OVX rats reduces endothelium-derived O_2^- production compared to untreated animals. Furthermore, we assessed the role of recently described non-phagocytic NAD(P)H oxidase (273;489) in the production of O_2^-, in particular its component $p47^{phox}$ (490). We also determined if changes in O_2^- production are associated with evidence of increased bioavailability of NO by examining the contractile and vasodilatory properties of rat aortic segments.

Atherosclerosis and Apoptosis

Injury of the vascular endothelium is a critical event in the pathogenesis of atherosclerosis (491). The atherosclerotic plaques develop preferentially in lesion-susceptible regions where turbulent blood flow prevails (492). Endothelial cells in regions were atherosclerotic abrasions preferentially develop demonstrate enhanced endothelial cell turnover rates (379) linked to programmed cell death or apoptosis (493). Distal parts of human atherosclerotic plaques show large areas of erosion characterized by increased apoptosis of inflammatory (494) and endothelial cells (495).

Apoptosis in endothelial cells (EC) is triggered by proapoptotic stimuli such as cytokines, adhesion molecules, reactive oxygen species (ROS) (496), high glucose, TNFα (380;497-499), oxidized low-density lipoproteins (oxLDL)(500) and aging (501;502). For instance, serum from patients with acute coronary syndromes increases the apoptosis of isolated HUVEC (503). Growth factors, laminar shear stress, nitric oxide, estrogen or

antioxidants suppress apoptosis (493). It has been demonstrated that E2 inhibits cytokine-induced apoptosis in human EC (394). Furthermore, E2 withdrawal from bovine endothelial cells in culture results in an increased rate of apoptosis (504). E2 can affect the nitrosation of the final effector of apoptosis, caspase-3. E2 also activates the PI(3)K/AKT survival pathway (505).

Oxidized LDL is a key element in triggering the earliest phase of atherosclerosis (29;506). OxLDL exposure increases the expression of adhesion molecules in human EC (507) and inhibits the endothelial regeneration after injury (508). It also increases proliferation and migration of vascular cells (509). Furthermore, oxLDL induces apoptosis in cultured human umbilical vein endothelial cells (HUVEC) (385;510). OxLDL has a dual effect on the cell cycle in HUVEC since it induces proliferation at low concentrations and apoptosis at higher concentrations through the induction of oxidative stress (511). The strong growth-promoting effect of oxLDL on human arterial smooth muscle cells (SMCs) plays a role in atherogenesis, whereas the induction of cell death by apoptosis leads to the development of an atherosclerotic lesion with necrotic foci (512). The cytotoxicity of oxLDL is especially important in the later stage of atherosclerosis for it renders atheromatous plaques unstable and prone to rupture (494).

The mechanism through which E2 decreases apoptosis in EC is not known. E2 was shown to activate multiple signaling cascades in bovine endothelial cells as well as breast cancer cells (513). It was also shown that the production of NO in HUVEC reduces their sensitivity to apoptotic stimuli (501). An increase of NO by E2 could also decrease endothelial sensitivity to apoptotic stimuli (501). E2 has been shown to decrease apoptosis induced by TNFα (394), by hypoxia (143) or by oxidative injury (496).

Therefore, in the present study we explored the mechanisms through which E2 decreases the apoptotic stimuli induced by clinically important agents TNFα and oxLDL.

Angiogenesis in Atherosclerosis

Chronic inflammation in the vasculature contributes to vascular injury and advances the atherosclerotic changes. In the early stage of atherogenesis, the inflammatory plaque is already associated with the formation of new vasa vasorum in areas of subsequent atherosclerotic plaque development (514;515). Eventually, most advanced atherosclerotic lesions are fairly neovascularized, especially those that are more vulnerable as a consequence of increased leukocyte infiltrations, local haemorrhage and tissue remodeling (516).

There are numerous angiogenic growth factors, namely VEGF, angiopoietins, but also TNFα, which stimulate EC proliferation, migration and survival, and mediate vascular hyperpermeability (517). TNFα was shown to be an inflammatory mediator because of its central place in the cytokine cascade, which was confirmed by the microarray analysis of the TNFα expression profile in HUVEC performed by collegues in our laboratory (518). The rationale is that when the endothelium is activated by inflammatory stimuli, the addition of E2 amplifies angiogenesis and the expression of prothrombotic proteins. Treatment with E2 could increase NO and decrease the expression of adhesion molecules. As we have also shown, E2 could decrease apoptosis in response to TNFα (519).

The main hypothesis is that in healthy women, E2 helps maintain a quiescent endothelium but when atherosclerosis and inflammation are present, the proangiogenic and prothrombotic effects of E2 worsen the vascular status.

The purpose of this study is to examine the harmful and beneficial effects of the interaction of E2 and TNFα on angiogenesis and inflammation in the endothelium. We hypothesize that the combination of E2 and TNFα in HUVEC results in increased angiogenesis and higher expression of prothrombotic molecules, but that it decreases the activation of adhesion molecules and also suppresses the increase in vascular permeability more importantly than treatment with TNFα alone. The degree of angiogenesis would correlate with VEGFR phosphorylation and Tie-2 phosphorylation.

II. Treatment with 17 β-estradiol reduces superoxide production in aorta of ovariectomized rats

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II.1. Abstract

Objective – Oxidant stress contributes to vascular injury and atherosclerosis. We hypothesized that estrogen treatment of ovariectomized rats decreases O_2^- by decreasing the activity of NAD(P)H oxidase and this reduction in O_2^- could have a vasculoprotective effect.

Methods and Results – Ovariectomized rats were treated with 17-β-estradiol (E2) (0.25 mg) or oil placebo for 21 days. Aorta was removed for contractility studies and O_2^- production was measured by lucigenin-enhanced chemiluminescence (230 and 5 μM). E2 treatment decreased basal O_2^- production but did not alter NADH or NADPH stimulated O_2^- production. Total $p47^{phox}$ and $p47^{phox}$ in membrane fractions of cardiac tissue were decreased, which suggests less activation of NAD(P)H oxidase in E2 treated rats. E2 did not change expression of other components of NAD(P)H oxidase in heart, lung, spleen and diaphragm. Expression of eNOS was also lower in E2 treated rats. E2 did not affect the contractile response to phenylepherine, dilation with acetylcholine, dilation with superoxide dismutase or constriction with L-NAME. This argues against changes in bioavailable NO.

Conclusions – E2 decreases activation of $p47^{phox}$ and O_2^- production by NAD(P)H oxidase. This did not affect contractile properties of the vessel, but could still potentially alter cell signaling from oxidant increasing stresses.

Key words; nitric oxide, hormone replacement therapy, vascular reactivity, chemiluminescence, oxidative stress, hormones

II.2. Introduction

The incidence of cardiovascular disease is lower in premenopausal women than men of similar age (19;520;521). Estrogen treatment also has protective effects in animal models of atherosclerosis and vascular injury (484) and mice with knockout of the estrogen α receptor have greater wall thickening in response to injury than animals with intact receptors (82). Although primary and secondary prevention trials in human subjects have failed to show a vascular protective effect of estrogen treatment in post-menopausal women (6;10;15), positive vascular effects of estrogen on the human vasculature are well documented, and the failure to find a positive effect may be because of a balance of positive and negative vascular effects of estrogen. Many of the positive vascular actions of estrogen are related to an increase in nitric oxide (NO) production (19;331;332) and the vasculo-protective effects of NO, including inhibition of expression of adhesion molecules and migration of inflammatory cells (71), improved flow mediated dilation (153), decreased apoptosis (501) and less expression of prothrombotic factors (19). Initially, it was thought that estrogen increased nitric oxide synthase (NOS) by increasing transcription through the estrogen response element (204), but expression of NOS is not increased in all studies (277;485). Subsequent studies have shown that estrogen increases NOS activity at least partly by phosphorylation of eNOS at serine 1177 (136-138), which increases eNOS activity. However, an increase in bioavailable NO can occur even without an increase in NOS activity and NO production, but by a decrease in superoxide (O_2^-) production (277;333). This occurs because O_2^- combines with NO and removes it by forming peroxynitrite, a potent oxidant which has further negative vascular affects (221). The objective of this study was to determine if estrogen

treatment of ovariectomized rats reduces vascular O_2^- production compared to untreated animals. Furthermore, we assessed the role of the newly described non-phagocytic NAD(P)H oxidase (273;489) in the production of O_2^- by examining the effect of diphenyleneiodonium (DPI), an inhibitor of flavin-containing enzymes and by adding NADH and NAD(P)H the substrates of this enzyme. NAD(P)H oxidase is a complex structure with multiple components. To be active, the components must assemble in the membrane. In phagocytic cells, $p47^{phox}$ is a key regulatory component (490). We therefore also examined the expression of total and membrane associated $p47^{phox}$. In addition, we determined if changes in O_2^- production are associated with evidence of increased bioavailablity of NO by examining the contractile and vasodilatory properties of rat aortic rings.

II.3. Methods

Animal Preparation

All procedures were approved by the ethics subcommittee of the University Animal Care Committee (see *Appendix 2)*. We used female Sprague-Dawley rats that were either intact or ovariectomized. Ovariectomy was performed by the supplier (Charles River Laboratories, St. Constant, QC) in 8-week-old animals (200 - 225 g) and shipped on the fifth day after surgery. A further 5 - 7 days were allowed for recovery. On day 14 after surgery, animals were anaesthetized with isoflurane and a hormone or placebo filled pellet (Innovative Research of America) was inserted subcutaneously in the neck. Pellets contained 0.25 mg of 17-β estradiol (E2) or placebo for active product, which was

released over 21 days. Animals were housed in pairs and allowed free access to food and water, in 12-hour day-night regime.

After 21 days, rats were sacrificed by intraperitoneal injection of 80 - 100 mg of sodium pentobarbital (MTC Pharmaceuticals). Organs were quickly dissected, frozen in liquid nitrogen and stored at -80°C for biochemical studies. For contraction studies, fresh 3 mm aortic rings were placed in 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. For the studies on vascular rings we also used male rats. These were larger (400-450 g) and older than the female rats. Male rats were not treated with hormones and the aortas were harvested as per the female rats.

Aorta rings bioassay

Vessels were cleaned and cut into ~3 mm segments and mounted horizontally between two stainless steel parallel hooks for the measurement of isometric tension (Grass-FT03 force transducer) and suspended in organ bath filled with 18 ml of Krebs-bicarbonate at 37°C and pH 7.4. The chamber was continuously oxygenated with 95% O_2 / 5% CO_2. After equilibration, resting tension and maximal contraction in response to 60 mM of KCl were determined. The resting tension of the rat aorta was ~2 g. Dose-response curves to increasing doses of phenylephrine (10^{-9} – 10^{-4} M) were then obtained and the response expressed as a percentage of the maximal response to KCl. The integrity of the endothelium was assessed by testing the vasodilatory response of preconstricted rings to acetylcholine (Ach, of 10^{-10} to 10^{-4} M). The dose-response to phenylephrine was repeated in vessels in which the endothelium had been removed by gentle rubbing. Vessels were

again preconstricted with 10^{-7} M phenylephrine and the vasodilatory response to SOD (1.5 U/ml) or constriction with L-NAME (10^{-8} to 10^{-4} M) was tested.

Chemiluminescence

Vascular segments were placed in Krebs-HEPES buffer and incubated in the dark for 10 minutes at 37°C. The tubes were transferred to a luminometer (Lumat LB 9501, Berthold Inc.) and 230 μM of lucigenin were added and the output of photons was measured for 7 minutes. The results with 230 μM were confirmed with 5 μM lucigenin to rule out artifacts from oxidative recycling of lucigenin (522). The background signal was subtracted from the total output and the area under the curve of output vs. time was calculated. We also measured the production of O_2^- by vessels incubated for 10 min at 37°C with 100 μM of NADH or NADPH, the substrates of NAD(P)H oxidase. A standard curve was obtained by measuring the output of O_2^- in a spectrophotometer by the reduction of cytochrome C by xanthine/xanthine oxidase (523;524).

Sources of O_2^- production, were assessed by pre-incubating rings for 10 minutes with 300 μM diphenyleneiodonium (DPI), an inhibitor of flavin-containing enzymes, 300 μM of N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide synthases or 3 mM of tiron, a cell permeable non-enzymatic O_2^- chelator.

Immunoblotting

Tissues were obtained from rat hearts, spleen, diaphragm, lung and aorta, and homogenised in lysis buffer (HB) (in a ratio of 5 ml of buffer/gram tissue). The buffer had the following composition (pH 7.5): 50 mM Hepes, 150 mM sodium chloride (NaCl),

100 mM sodium fluoride (NaF), 10 mM tetrasodium pyrophosphate ($Na_4 P_2O_7 .10H_2O$), 5mM disodium ethylene diamine tetraacetate (EDTA. $2H_2O$), 0.5% Triton X-100, 0.5% deoxycholic acid ($C_{24}H_{39}O_4Na$), 5 mg/ml aprotinin, 2 mg/ml leupetin, 1 mg/ml pepstatin, 100 mM activated sodium orthovanadate (Na_3VO_4). Homogenates were centrifuged at 2000x g for 30 minutes at 4°C, the bottom pellet was discarded. The supernatant was resolved by SDS-PAGE and transferred to PVDF membranes. Protein was measured by the Bradford method. Membranes were incubated with monoclonal antibodies to $p22^{phox}$, $p47^{phox}$, $p67^{phox}$ (gift from Genentech), eNOS (Transduction Laboratories) and polyclonal $gp91^{phox}$ (gift from MR Quinn, Montana State University). For detection, membranes were incubated in chemiluminescence solution (Lumi-Light Plus, Roche) and visualized by autoradiography (films Biomax-MR, Kodak). The blots were scanned with an imaging densitometer and optical densities of protein bands were quantified with SigmaGel software (Jandel Scientific Inc.).

Immunoprecipitation

The protein concentration of samples was adjusted to 1 mg per 0.5 ml with HB and then 5 µl of anti-$p47^{phox}$ monoclonal antibody (Genentech) was added and the mixture incubated for 12 hours. Next we added 25 µl of protein A/G-agarose conjugate (Santa Cruz Biotechnology) for 3 hours at 4°C. Immunoprecipitates were pelleted by microcentrifugation at 10,000 rpm for 5 minutes. For electrophoresis, 15 µl of supernatant sample was added to 15 µl of loading buffer prepared from sample buffer (Invitrogen) with 5% β-mercaptoethanol. Pelleted beads were resuspended and washed with 0.5 ml of ice-cold immunoprecipitation HNTG-buffer with the following

composition (pH 7.5): 20 mM Hepes, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, then microcentrifuged at 5,000 rpm for 5 minutes. This supernatant was discarded after washing the beads. The pellet was washed in HNTG-buffer and centrifuged three times). After the final wash, the pellet was resuspended in 15 µl of electrophoresis loading buffer. All immunoprecipitation procedures were done on ice. Crude sample, pellet, and supernatant were resolved on SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes for immunostaining.

Preparation of membrane and cytosolic fractions

To determine cytosolic and membrane contents of $p47^{phox}$, we added ice-cold buffer without deoxycholic acid to cardiac tissue and ground it in a blender until fully homogenized. The homogenate was centrifuged at 2,000 g for 20 minutes at 4°C and the supernatant was saved. Nuclei and unbroken cells were centrifuged at 2,900 g for 15 minutes at 4°C. Supernatant, which contains cytosol and membrane fractions, was ultracentrifuged at 29,000 g for 45 minutes at 4°C. Supernatant from this step was saved as a cytosolic fraction. The remaining pellet was resuspended and ultracentrifuged at 15000xg for 20 minutes. The supernatant from this step was saved as the membrane fraction and run on SDS-PAGE and transferred to a PVDF membrane.

Northern Analysis

An eNOS cDNA probe was created for Northern analysis by first amplifying a fragment by polymerase chain reaction, ligating the sample with T-4 DNA ligase into

PGEM-T vector and transforming competent E-coli cells. The probe was then labeled with alpha[^{32}P] deoxycytidine triphosphate by random priming.

For Northern analysis, total RNA (50 μg) samples from the aorta of rats treated with E2 100 μg /kg, progesterone 1 mg/kg, E2 plus progesterone and E2 plus tamoxifen and E2 receptor antagonist were fractionated by electrophoresis on a denaturating formaldehyde 1% agarose gel and transferred to Hybond-N nylon membrane. RNA was immobilized and membranes were prehybridized for 3 hrs at 42°C with 5 x SSPE, 5 x Denhard's solution, 0.5% SDS, 50% formamide and 10 μg/ml of a heat denatured salmon sperm DNA. Hybridization was done overnight in a solution containing ^{32}P labelled eNOS probe. The membranes were exposed to film at -80°C.

Radioimmunoassay for estrogen

Serum estradiol was measured with a competitive immunoassay using direct chemiluminescent technology. The assays were performed on the ACS/centaur automated analyzer (Chiron Diagnostics).

Statistics

Data are presented as mean ± SEM. Paired data were analyzed with a t-test. Multiple comparisons were performed with ANOVA and repeated measures with multiple conditions, as in the contractile studies, were analyzed with a two-way ANOVA for repeated measures. Statistical significance was accepted at $p < 0.05$.

II.4. Results

Estrogen treatment increased uterine weight from 0.14 ± .05 g in untreated animals to 0.57 ± .12 g in E2 treated animals (*Fig. 1 A*). E2 treatment was associated with a decrease in body weight from 323.3 ± 19.5 g in control ovariectomized animals to 269.6 ± .12 g in E2 treated animal ($p < 0.001$) (*Fig. 1 B*). Plasma estradiol was 49 ± 18 pmol/l in untreated rats, which is in the range of post menopausal women, and 454 ± 185 pmol/l in rats treated with E2 (*Fig. 1 C*), which is in the range of the luteal phase of normal women (42) and therefore physiologic.

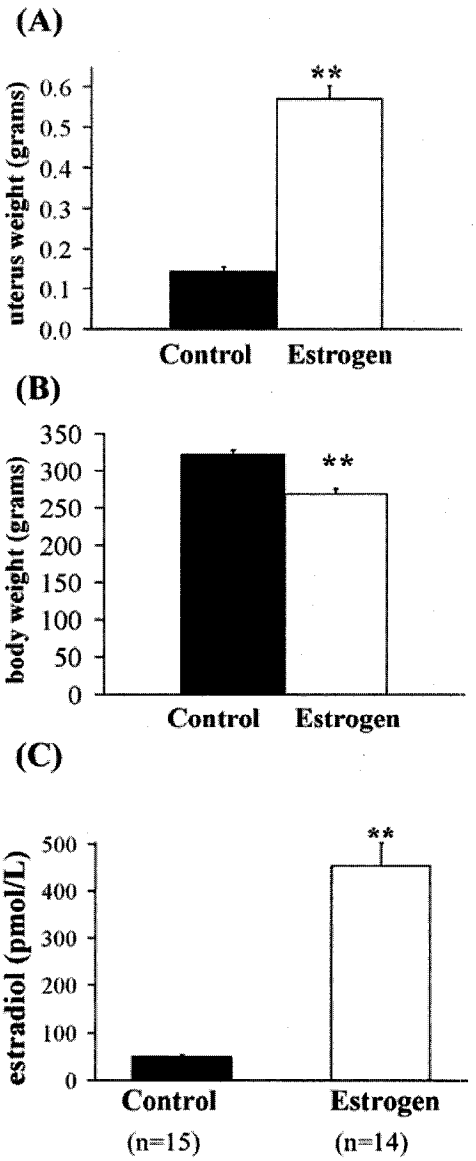

Figure II-1. Serum estradiol concentration, body and uterus weight after 21-day treatments.

A. Uterus weight in ovariectomized rats implanted with placebo pellets (Control) compared to rats treated with estradiol. **B**. Body weight is higher in untreated, placebo-pellets implanted ovariectomized rats (Control) than in E2 treated rats. **C**. Serum estradiol concentration in E2 treated vs. placebo treated female rats. Control (n=15), Estrogen (n=14), by *t*-test ** P=<0.001.

Superoxide production

Estrogen treatment lowered O_2^- production in rat aorta (n=12 pairs, control and E2 treated) (*Fig. 2 A*). The same pattern was seen with 5 μM of lucigenin as with 230 μM (n=3 pairs) (*Fig. 2 B*). The NAD(P)H inhibitor, DPI, decreased O_2^- production in untreated animals, but not in animals treated with E2 (*Fig. 2 C*) whereas L-NAME had no effect on either group.

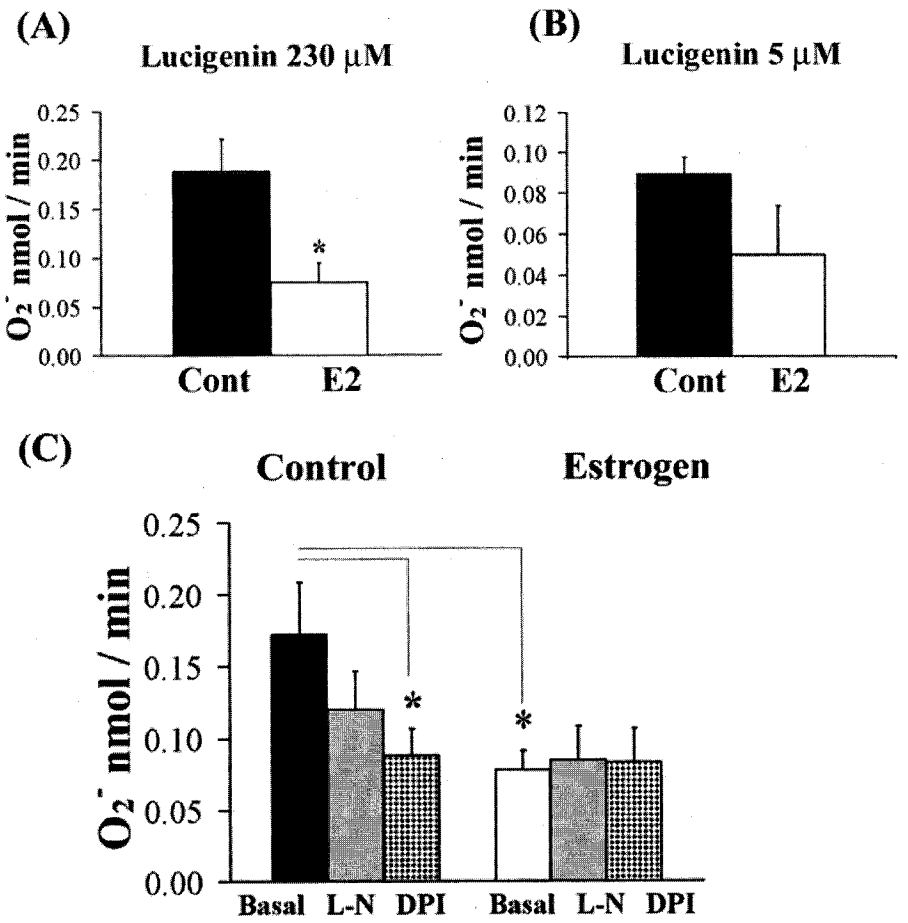

Figure II-2. Production of superoxide by aortic rings as determined by lucigenin-enhanced chemiluminescence.

A. Production of O_2^- in nmol/min with 230 μM lucigenin (n=12) (left) was significantly reduced. **B**. The pattern was similar with 5 μM lucigenin (n=3) Cont refers to control and E2 to 17-β-estradiol. **C**. L-NAME (L-N) had no significant effect on O_2^- production in either control or E2 animals. DPI lowered O_2^- production in control but it had no effect in E2 treated animals because the O_2^- production was already low.

Incubation of rat aortic rings with NADH increased O_2^- production more than 100 fold, and there was no difference in E2 treated (n=8) vs untreated rats (n=9) (*Fig. 3*). NADPH also increased O_2^- production, but the increase was only a third of that seen with NADH, and again there was no difference between treated and untreated rats. The O_2^- scavenger, tiron, markedly suppressed the increase in O_2^- seen with both NADH and NADPH. The nitric oxide inhibitor, L-NAME, had no effect.

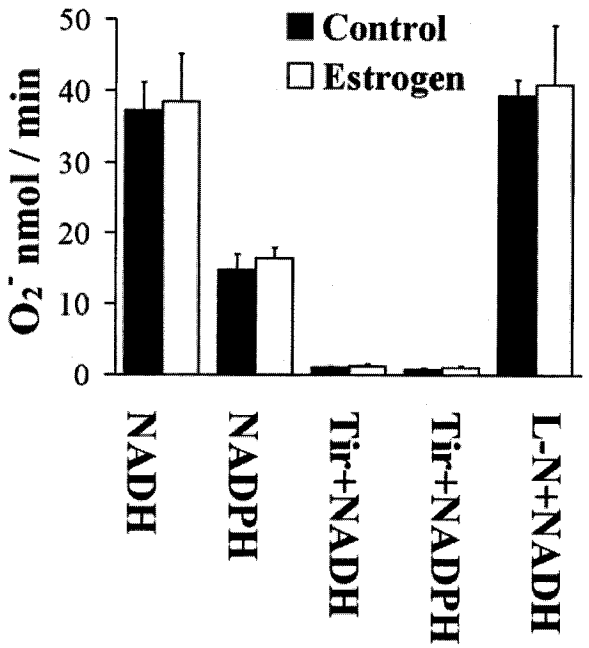

Figure II-3. NADH and NAD(P)H stimulated production of superoxide by aortic rings.

(control n=9, estrogen n = 8). NADH markedly increased O_2^- production and so did NADPH, but to a smaller extent than NADH. Note the difference in scale from Figure 2. Tiron almost completely inhibited O_2^- production produced by NADH and NADPH. L-NAME had no effect. There was no difference between control and E2 animals for any condition.

Western analysis

There was no evident change in expression of $p22^{phox}$ in spleen and cardiac tissues. The $p67^{phox}$ component was studied in spleen, heart and lung and there was no evident regulation (data not shown). Changes in $p47^{phox}$ were studied more extensively in cardiac tissue. E2 reduced overall expression of $p47^{phox}$ in crude samples (example in *Fig. 4 A*) (density of 1147 ± 525 vs 568 ± 186 in estrogen treated, n=10, $p< 0.05$). E2 decreased the expression of $p47^{phox}$ in the membrane fraction (*Fig. 4 B*), but there was no evident change in the cytosolic fraction (*Fig. 4 C*).

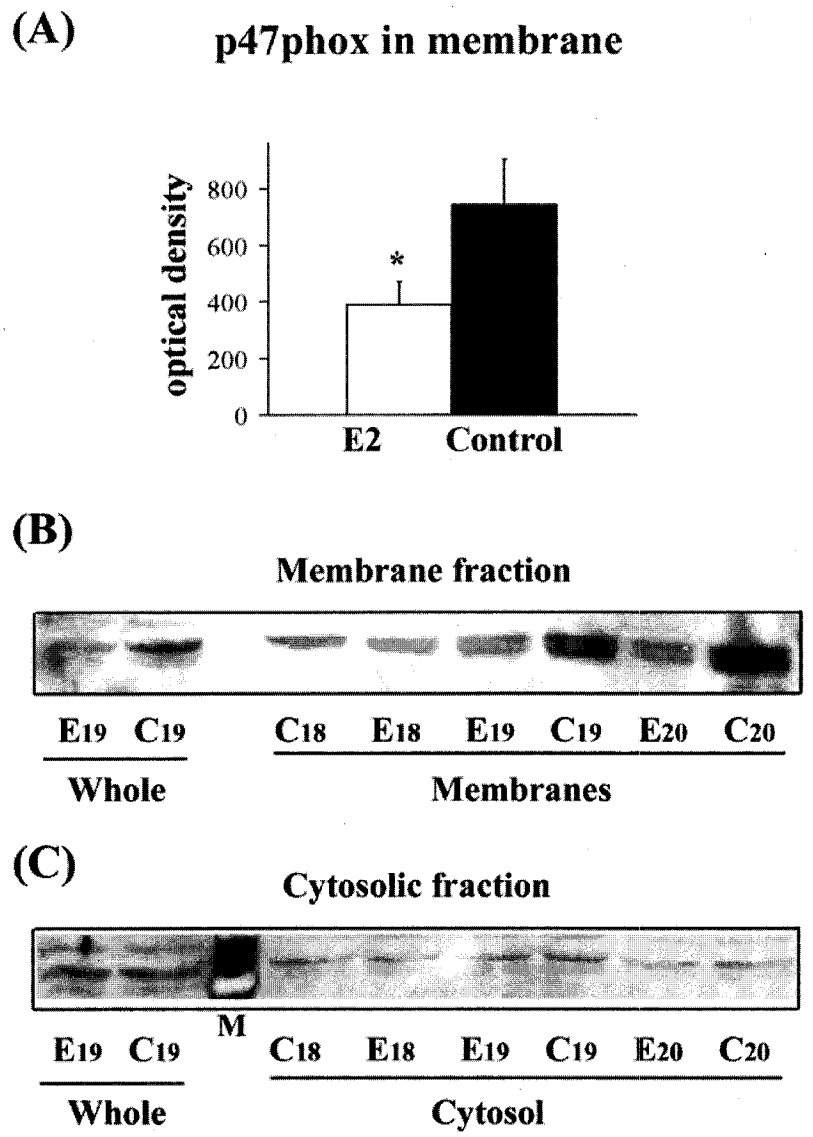

Figure II-4. Immunoblot of 47^{phox} expression in hearts from ovariectomized rats treated with E2 or untreated.

A. Expression of p47phox in unfractionated tissue. Expression of $p47^{phox}$ was lower in E2 treated than in untreated animals (Cont) ($p< 0.04$). **B**. Examples of expression of $p47^{phox}$ in membrane fraction of cardiac tissue. Membrane expression of $p47^{phox}$ in treated rats (E) was less than in untreated rats (C). "Whole" refers to unfractionated samples. Animals were studied in pairs and the numbers refer to sample animal pairs. **C**. Expression of $p47^{phox}$ in cytosolic fraction. There was no evident change in the cytosolic fraction with E2 treatment.

In contrast to what we predicted, when we immunoprecipitated $p47^{phox}$ to concentrate the sample, more $p47^{phox}$ was immunoprecipitated in E2 treated than untreated animals (*Fig. 5 A*), but there were only small differences in the supernatant (*Fig. 5 B*).

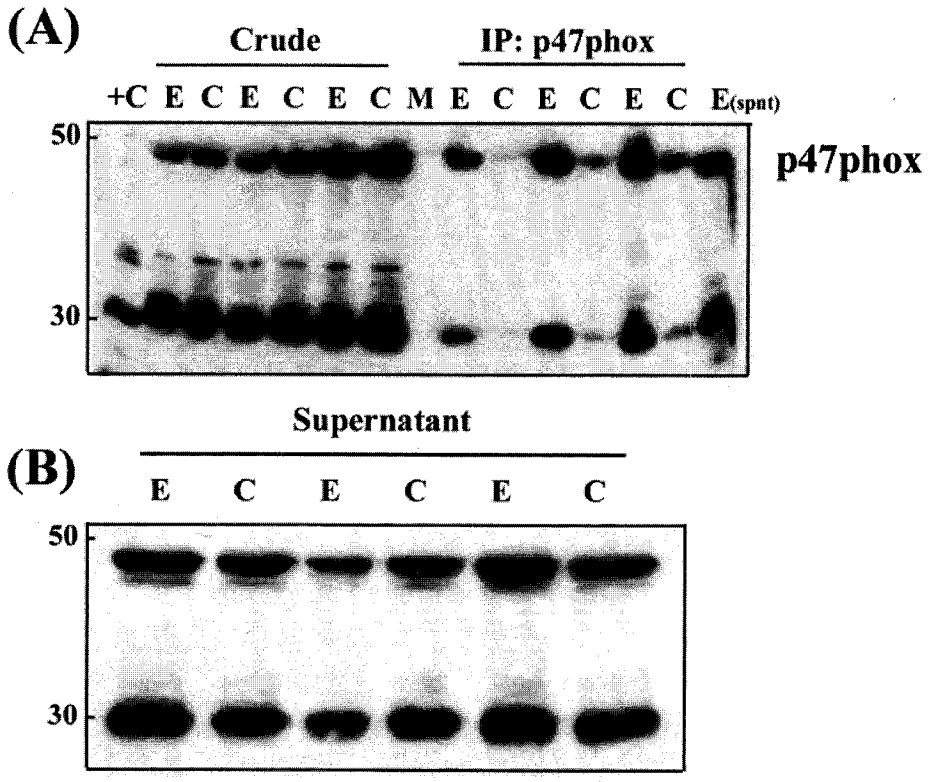

Figure II-5. Immunoblot of $p47^{phox}$ immunoprecipitated (IP) from cardiac tissue of ovariectomized rats.

A. Crude and IP samples from estrogen treated (E2) or untreated (C). Animals were treated in pairs and samples were placed on the gel accordingly. As in figure 4, E2 treated rats had lower $p47^{phox}$ expression in the crude sample of each pair. However, in contrast, by IP $p47^{phox}$ expression was much lower in control rats compared to E2-treated rats. **B.** $p47^{phox}$ expression in the supernatant from the IP. Expression of $p47^{phox}$ in C tended to be lower than E2 treated rats but difference was much smaller than in the IP fraction.

Aortic ring studies

E2 did not change contractions induced by phenylephrine as a percentage of that produced by KCL (*Fig. 6 A, B*). We included untreated male rats (n=9) in this protocol; their curve was shifted to the left of that of female rats (n=6). Removing the endothelium did not alter the response, indicating that the difference in the response between males (n=9) and females (n=6) was not endothelial-dependent. Incubation of the rings with SOD (*Fig. 6 C, D*) produced the greatest dilation of partially constricted aortic rings in untreated females (untreated females, n=6; E2 treated females, n=6; males, n = 8). SOD-induced dilation analyzed by groups was reduced after removal of the endothelium ($p<0.001$ by ANOVA for repeated measures), but differences between endothelial present or not present were only significant for male rats and oil-treated females by post hoc analysis ($p<.001$).

The dose response to acetylcholine in pre-constricted aortic rings of male rats (n = 9) was shifted to the left of the female rats (n=6), but there was no difference in treated or untreated females (n=6) (*Fig. 7 A*). There was greater constriction when L-NAME was added to partially constricted aortic rings in female rats compared to male rats, but again there were no differences between E2-treated and untreated rats (*Fig. 7 B*).

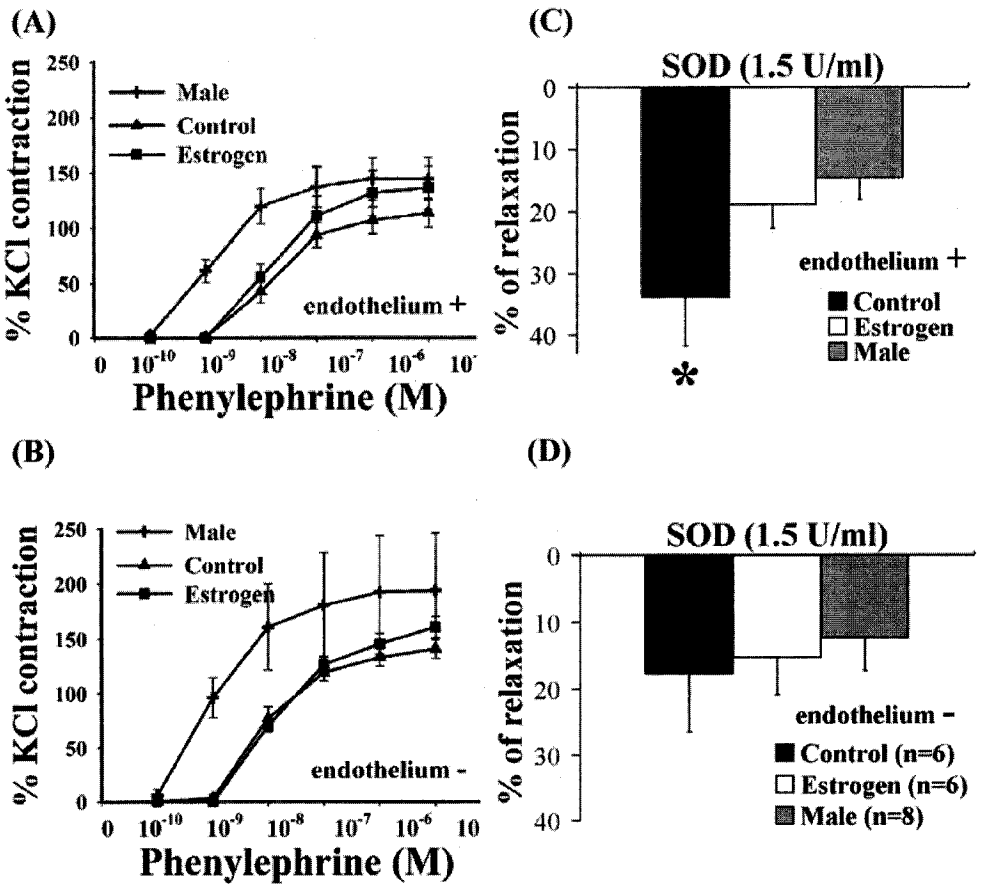

Figure II-6. Contractile responses to phenylephrine and relaxation with superoxide dismutase (SOD) in rat aorta.

A. Contractions with increasing doses of phenylepherine with the endothelium intact. **B**. Contractions with the endothelium removed. Values were normalized to the contraction produced by KCl. The response in male rats (┼) was shifted to the left of that of ovariectomized rats and reached a higher plateau. Treatment of ovariectomized rats with E2 (■) did not alter the contractile response compared to untreated rats (▲) with or without the endothelium. **C**. A bar graph of the SOD-induced dilation of rings preconstricted with phenylepherine (10^{-7} M) with endothelium intact and in D with the endothelium removed. Untreated rats tended to have greater dilation than E2-treated or male rats but the difference was not significant. The group response in C (endothelium intact) was significantly greater than in **D** (endothelium removed) (by two-way ANOVA). By post hoc analysis, only the differences between control and males in the two conditions were significant.

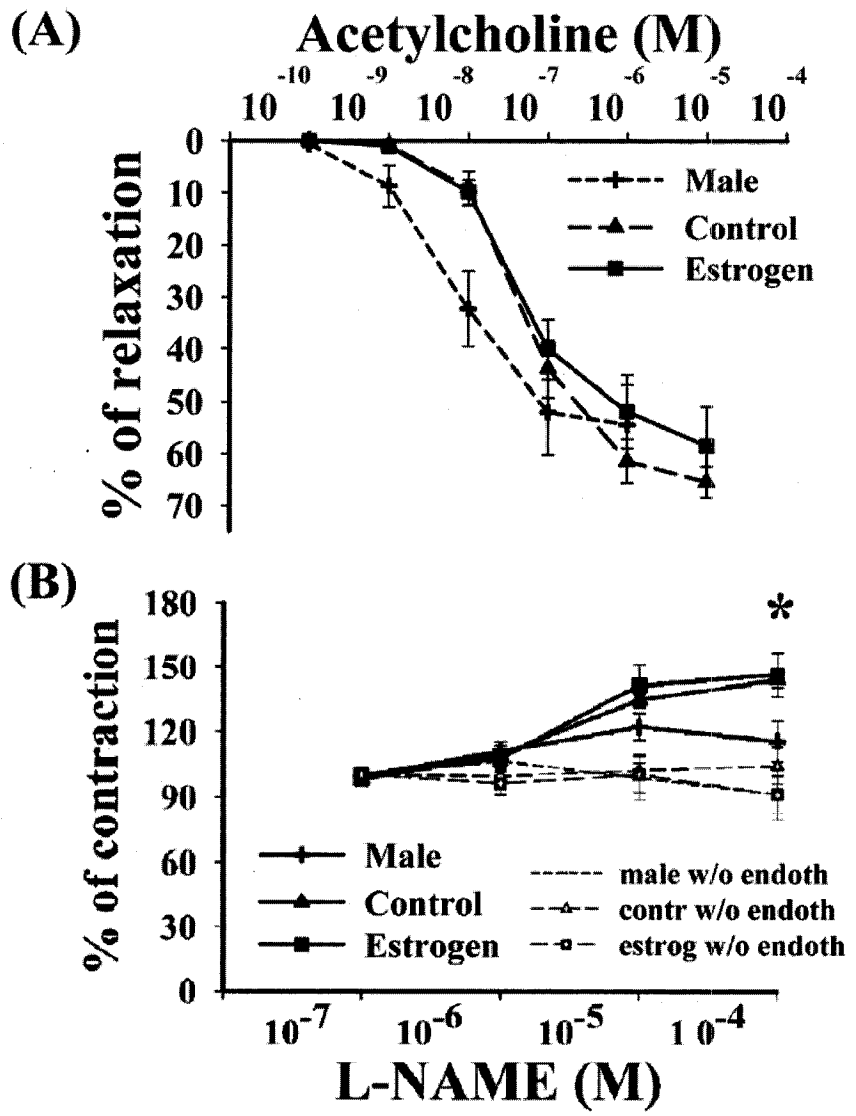

Figure II-7. Endothelial-dependent dilation and constriction of aortic rings from rats. **A**. Ovariectomized rats with intact endothelium had the same vasodilatory response to acetylcholine with (■) or without (control, ▲) treatment with E2. The response in male rats (┼) was shifted to the left. **B**. L-NAME produced greater vasoconstriction in ovariectomized rats than male rats (┼) and the response was not difference in estrogen treated (■) or not treated (▲). Stimulations in similar treatments but in endothelium removed rat aorta are represented by dashed thin lanes with corresponding symbols.

Endothelial NOS expression

By Western analysis, eNOS expression was actually lower in the aorta of E2 (n=5) treated compared to untreated rats (n=5) as well as intact female rats (n=3) ($P<0.05$) (*Fig. 8 B ,C*). As part of a preliminary study we also measured eNOS mRNA expression by Northern analysis in aorta and hearts from ovariectomized rats treated with placebo, E2, progesterone, E2 plus progesterone, and E2 plus tamoxifen (*Fig. 8 A*). There was no difference between any of the group; if anything the expression of eNOS in the aorta of E2 treated animals was less than in oil treated control animals consistent with observations on Western analysis.

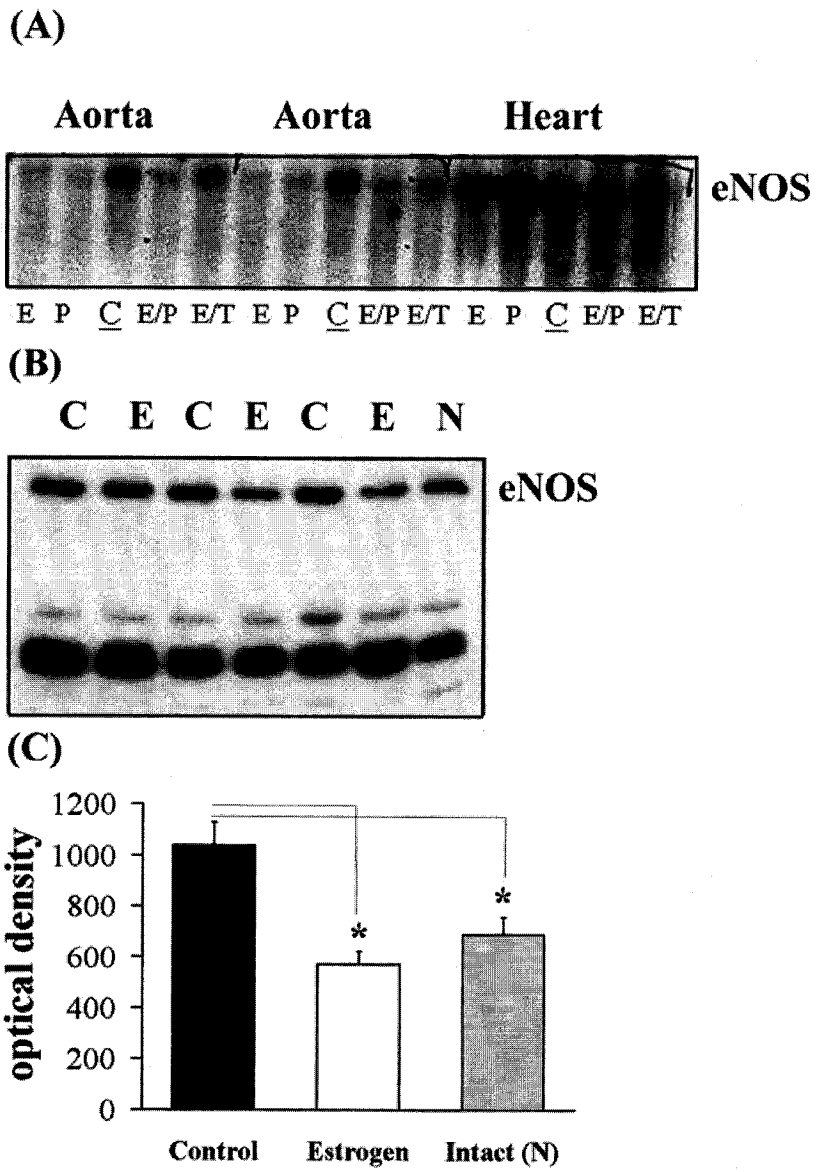

Figure II-8. Expression of eNOS in rats with and without E2 treatment.

A. mRNA by Northern analysis in aorta (n=2) and a heart of rats. E is treatment with E2, P is progesterone, C is the control, E/P is E2 plus progesterone, and E/T is estrogen plus tamoxifen. Expression in the aorta tended to be higher in control animals. In the heart, there was no difference among the conditions. **B**. Protein expression by western analysis in aorta from 5 control, 5 E2 treated and 3 intact female rats. **C**. Average densities are shown in the histogram. eNOS expression was higher in untreated ovariectomized rat (C) than in the E2-treated animals (E) or intact females (N for normal).

II.5. Discussion

We found that O_2^- production was lower in the aorta of ovariectomized rats treated for three weeks with E2 than in untreated animals as has been observed recently by others (338;525). We also found that DPI inhibited O_2^- production, which supports a role for NAD(P)H oxidase in the production of O_2^-. However, E2 did not alter NADH or NAD(P)H stimulated O_2^- production, which suggests that the amount of NAD(P)H oxidase did not change. The decrease in O_2^- production with E2 was associated with a decrease in the $p47^{phox}$ component of NAD(P)H oxidase, and in particular, the membrane component of $p47^{phox}$, which supports the hypothesis that the regulatory effect of E2 occurs through alterations in $p47^{phox}$. The decrease in O_2^- production was not associated with a change in the contractile response to phenylephrine or dilation in response to acetylcholine or SOD, which argues against an increase in bio-available NO. Finally, eNOS expression was not increased by the treatment and if anything decreased.

The production of reactive oxygen species, and O_2^- in particular, has been associated with vascular disease. In endothelial cells, increased O_2^- production decreases NO bioavailability, activates pro-atherosclerotic pathways and results in endothelial dysfunction (294;526). A potentially important source of O_2^- production in vascular tissues is the recently described non-phagocytic form of NAD(P)H oxidase (273;527;528). This enzyme complex is made up of at least six components, including $p22^{phox}$, $gp91^{phox}$, $p67^{phox}$, $p47^{phox}$, $p40^{phox}$ and small G proteins Rac and Rap1a and 2 and these components appear to be the same as those in phagocytic cell. The enzyme has been identified in aortic walls (523;524) and vascular smooth muscle cells (296) where its activation is involved in the vascular hypertrophy produced by angiotensin II

(296;529-531). It is also present in endothelial cells where its role is less clear (317;336;490).

We based our studies on the observation by Arnal and co-workers (277) who found that estrogen increased bio-available NO in endothelial cells without changing the expression of eNOS. They hypothesized that this could be due to decreased production of O_2^-, which would result in more bio-available NO, for it has long been known that O_2^- decreases bio-available NO (246;532). They found that E2 decreased O_2^- production, but did not have a mechanism for this. In a subsequent publication (333), they showed that estrogen treatment decreases basal vascular O_2^- production. They also found that E2 shifted the aortic endothelial dependent vasodilation leftward, which is consistent with more available NO.

We hypothesized that estrogen reduces O_2^- production by decreasing the activity of NAD(P)H oxidase. Since activation of this complex requires the mobilization of $p47^{phox}$ and other cytosolic components to the membrane in phagocytic cells, we thus also hypothesized that regulation of $p47^{phox}$ is involved in the estrogen effect. In support of these hypotheses, basal O_2^- production was decreased and so was total $p47^{phox}$ expression and, more importantly, $p47^{phox}$ in the membrane.

Wagner et al (336) also studied the effect of E2 on NAD(P)H oxidase in endothelial cells. They found that E2 did not change basal O_2^- production, but E2 decreased phorbol ester induced O_2^-production. Decreased O_2^- production was associated with decreased expression of adhesion molecules and chemokines in response to cyclic strain (336). They proposed that the decreased O_2^- production was based on decreased expression of components of NAD(P)H oxidase including $gp91^{phox}$, $p22^{phox}$ and $p47^{phox}$.

In contrast, we found no change in $p22^{phox}$ and $p67^{phox}$ expression in the two tissues examined and recently Gragasin et al (337) found that E2 actually increased expression of $gp91^{phox}$ protein in bovine microvascular cells.

As already noted, in our study, total $p47^{phox}$ was decreased in the hearts of E2 treated rats compared to untreated animals, especially in the membrane component. This suggests that E2 reduces the attachment of $p47^{phox}$ to the membrane bound components, which is believed to be important for activation of the complex. A surprising finding was that despite there being a lower concentration of total $p47^{phox}$, more $p47^{phox}$ was immunoprecipitated in E2-treated than untreated rats. This suggests that the epitope for the monoclonal antibody was less available in untreated animals, perhaps because more was attached to the NAD(P)H oxidase membrane complex. This could have occurred because of an E2 induced conformational change in $p47^{phox}$ when it is phosphorylated and activates the full NAD(P)H oxidase complex (533) Thus, even the reduction in total $p47^{phox}$ may not represent a true decrease but rather a decrease in the immunologically available protein.

It has recently been shown that decreased O_2^- production with E2 treatment in aorta of rats and vascular smooth muscle is associated with decreased Rac-1, a GTPase that is involved in the assembly of NAD(P)H oxidase (338). This is thus an alternative or additional way that E2 could regulate NAD(P)H oxidase.

Stimulation of NAD(P)H oxidase by providing the substrates, NADH or NAD(P)H, did not alter O_2^- production in E2-treated compared to untreated rats. Since this stimulation tends to test the maximal capacity of the enzyme, it indirectly reflects the amount of enzyme present and the failure to see an E2 effect supports the argument that

there was no significant change in expression of the components of the enzyme by E2 treatment of ovariectomised rats.

Wagner et al (336) found that ovariectomy decreased eNOS expression, whereas we found no change in eNOS expression by both Northern and Western analysis. Our findings are consistent with those of Arnal and co-workers (277;333). Our studies on vascular rings also argue against a change in NOS expression or bioavailable NO. As also observed by Barbacanne et al (333), E2 did not affect the contractile response to phenylephrine. However, in contrast to their study, we also found no difference in the vasodilatory response to acetylcholine. The contractile response to phenylephrine was greater in male rats than in female rats and was not affected by removal of the endothelium, which also argues against a change bio-available NO. The greater contractile response in males than in females could have been related to their larger size.

We expected that treatment of vascular rings with SOD could uncover a reduction of bio-available NO in untreated animals as observed by others (257), but the analysis is confounded by changes in both eNOS activity and O_2^- production. SOD treatment produced the greatest dilation in untreated ovariectomized females, consistent with a larger production of O_2^-. Removal of the endothelium greatly reduced the SOD induced dilatation in untreated rats, which supports an endothelial source for the O_2^-. Somewhat in contrast to our results, Hayashi et al (257) found that intact female rabbits had greater dilation with SOD treatment than male rabbits or ovariectomized rabbits. Differences between our studies could be because our "intact" animals were treated with E2, and also because they used rabbits whereas we used rats.

The failure to see a leftward shift in the acetylcholine dose response curve (ie increased sensitivity) after E2 treatment at first seems to contradict a number of studies in which estrogen treated vessels from both animals and humans were studied (84;214). We, too, previously have found a leftward shift of the acetylcholine dose response curve (138) and attributed it to activation of an AKT/PKB pathway as identified by others (17;137). The difference may be that leftward shifts of the vasodilatory response have been demonstrated in acute studies, whereas in this study, animals were examined after three weeks of treatment. This may mean that compensatory mechanisms are activated over time.

A failure to find a difference in vascular responses in chronically treated versus untreated rats might be explained by production of endogenous estrogens in untreated animals. Ovariectomized rats gain weight after recovering from the surgery which means that there is more peripheral tissue which can make E2 by the conversion of adrenal androgen precursors to estradiol through the action of aromatase (534). Indeed, we also found that the weight of untreated ovariectomized rats was higher than the weight of E2 treated rats. However, this cannot explain our results because E2 treated animals had almost ten times the estrogen concentration of untreated animals. An estrogen effect also was evident by the much higher uterine weights treated versus untreated animals.

II.6. Conclusions

Chronic estrogen treatment of ovariectomized rats decreased the production of O_2^- by the vascular wall, but this was not associated with evidence of increased bio-available NO or a change in vascular contractile properties. The decrease in O_2^- production was

associated with a decrease in the membrane fraction of $p47^{phox}$, which suggests that estrogen treatment alters O_2^- production by regulating the association of $p47^{phox}$ with the full NAD(P)H oxidase complex. It is possible that the estrogen-induced decrease in O_2^- production could alter intracellular signaling and thereby contribute to the vascular protection observed with estrogen treatment.

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Bridge between the first and the second manuscript.

Reactive oxygen species (ROS) are known to induce apoptotic cell death in various cell types, including endothelial and smooth muscle cells (535). Apoptotic cell death may play a role in the initiation of atherogenesis (493). Enhanced production of ROS in the vascular endothelium is a very early hallmark in the atherogenic process, even preceeding atherosclerotic lesion formation (536), suggesting a link between ROS and EC apoptosis. It was shown that exogenous ROS such as H_2O_2 or O_2^- can trigger apoptosis of ECs (383;537). Various pro-atherosclerotic factors such as oxidized LDL, are known to induce the endogenous synthesis of ROS in ECs and to activate the apoptosis execution program in ECs (385;510). Finally, the proinflammatory cytokine TNFα induces EC apoptosis via oxygen radical formation (538). Inhibitor of the ROS-generating NAD(P)H oxidases, the flavin protein inhibitor DPI or an antioxidant the N-acetylcysteine, protect ECs against programmed cell death (539). Thus, exogenous ROS or stimulation of ROS formation in ECs by proatherosclerotic or pro-inflammatory factors promote apoptotic cell death of ECs.

On the other hand, endothelial cells are exposed to protective factors that stimulate anti-apoptotic pathways. Anti-atherosclerotic functions of endothelial NOS are well known. Endothelium-derived NO inhibits EC apoptosis induced by oxLDL, angiotensin-II, TNFα and ROS (540).

E2 is known to restore normal endothelial function in humans and animals with atherosclerosis, particularly through the increase in NO production and the reduction of expression of adhesion molecules in response to cytokine stimulation (71;331;332;484).

Thus, we hypothesized that E2 decreases ROS, notably O_2^-, by decreasing expression of non-phagocytic NAD(P)H oxidase. We hypothesized that E2 would also reduce apoptosis in EC triggered by oxidized LDL and proinflammatory cytokine TNFα. Consequently, these effects of E2 contribute to the decrease of endothelial injuries and increased endothelial health.

III. ESTROGEN DECREASES TNFα AND OXIDIZED LDL INDUCED APOPTOSIS IN ENDOTHELIAL CELLS

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III.1. Abstract

Apoptosis induced by oxidized low-density lipoproteins (oxLDL) and tumor necrosis factor-α (TNFα) is believed to contribute to atherosclerosis and vascular dysfunction. Estrogen treatment reduces apoptosis due to TNFα and we hypothesized that it would also reduce apoptosis due to oxLDL. We also explored the antiapoptotic mechanisms.

We used early passage human umbilical vein endothelial cells (HUVEC) grown in steroid depleted, red phenol-free medium. Cells were synchronized by starvation for 6 hours and then treated with oxLDL (75 μg/ml) or TNFα (20 ng/ml) in the presence of 17-β-estradiol (E2) (20nM). Apoptosis was analyzed by flow cytometry and caspase-3 cleavage. We also assessed expression of Bcl-2 and Bcl-xL and phosphorylation of BAD.

At 6 hr TNFα induced apoptosis but oxLDL did not; E2 did not affect this TNFα induced apoptosis and there was no change in Bcl-2 or Bcl-xL expression. At 24 hr both TNFα and oxLDL increased apoptosis and E2 reduced the increase. E2 also increased expression of the antiapoptotic Bcl-2 and Bcl-xL and increased phosphorylation of proapoptotic BAD which reduces its proapoptotic activity at 1 hr. However at 24 hr there was also an increase in total BAD so that the proportion of phosphorylation of BAD decreased.

oxLDL induced apoptosis occurs later than that of TNFα. E2 decreased this late phase apoptosis and this likely requires the production of antiapoptotic proteins.

Key words: apoptosis, atherosclerosis, endothelial cells, estrogen, oxidized low-density lipoprotein, tumor necrosis factor-α

III.2. Introduction

The place of estrogens in women's health remains controversial. The incidence of atherosclerotic illness in women increases markedly after menopause and in observational studies, post-menopausal women who took estrogen replacement therapy had a lower incidence of cardiovascular disease than women who did not (2;19;484;520;521;541;542). These observations in humans were supported by studies in primates in which estrogen was antiatherogenic (194) and rodents in which estrogen accelerated vascular healing (191). However, in randomized primary and secondary cardiovascular prevention trials in humans, estrogen replacement therapy did not reduce cardiovascular events and there was even a suggestion of harm (6;10;17;22;543). To better predict these contrasting negative and positive vascular effects of estrogens and thus to better define the proper place for estrogen replacement therapy in the management of postmenopausal women, it is necessary to have a better understanding of the action of estrogen on the vascular wall. An important activity for the normal function of the vascular wall is the regulation of apoptosis of endothelial cells.

Apoptosis is now recognized as playing a fundamental role in the pathobiology of atherosclerosis (544). Endothelial cell apoptosis is increased by hypoxia (143), oxidative stress (496), nitrosative stress (500), cytokines (394), oxidized low-density lipoprotein (oxLDL) (500), and aging (501;502). Apoptosis is suppressed by shear stress and therefore is lower in areas of normal flow and higher in areas of reduced laminar flow (385;544;545). Serum of patients with heart failure increases apoptosis of endothelial cells in culture (503). Adhesion of platelets and inflammatory cells is greater on apoptotic

endothelium and denuded vessel walls are thrombogenic. Since estrogens are also pro-thrombotic, estrogen administration to women who have injured endothelium and increased endothelial apoptotis could result in increased thrombotic events. On the other hand, estrogens have been shown to decrease endothelial cell apoptosis, which potentially could provide a cardiovascular protective effect. For example estrogen treatment decreased apoptosis induced by TNFα (394), hypoxia (143), and oxidative injury (496) and estrogen withdrawal increased apoptosis (504). The net effect of estrogen in the intact person would depend on the magnitude of these positive and negative effects of estrogen.

Because of the importance of oxLDL in the atherosclerotic process we investigated the effect of estrogen on apoptosis induced by oxLDL in human umbilical vein endothelial cells (HUVEC). We compared the effect of estrogen on oxLDL induced apoptosis to that induced by TNFα for the apoptotic response to TNFα has been previously characterized (394) and could serve as a reference value. We also investigated the effect of estrogen on the time course and factors that regulate apoptosis induced by these two agents.

III.3. Materials and Methods

Cryopreserved HUVEC were purchased from GlycoTech (No.41-001, Gaithersburg, MD) or Cambrex Bio Science (CC-2519, Walkersville, MD). Cell culture media and supplements were obtained from Invitrogen unless otherwise stated. Subsequent components were obtained as follows: charcoal stripped de-lipidated fetal bovine serum (C-1696), gelatine type B solution (G-1393), magnesium sulfate solution (M-3409),

sodium selenite (S-9133), human apo-transferrin (T-5391), bovine serum albumin (A-8806), TNFα (T-6674), camptothecin (C-9911) and endothelial cell growth supplement (E-2759) from Sigma, endothelial mitogen (ECGS) from Biomedical Technologies (BT-203, Stoughton, MA), lyophilized 17-β-estradiol (E2) from Sigma (E-2257) or in powder form from Steraloids (E0950-000, Newport RI), human oxidized low-density lipoprotein from Intracel (RP-038, Frederick, MD), PeroxiDetect kit (PD-1) from Sigma, ICI 182,780 from Tocris Cookson (No.1047, Ellisville, MO), propidium iodide powder (P-4170) or solution (P-4864) from Sigma, RNase A from Quiagen Sciences (No.19101), Falcon tubes (No.352008, Becton Dickinson).

Primary antibodies for western blotting were from following companies: cleaved caspase-3 (Asp175) (N0.9661), Bcl-xL (No.2762), phospho-BAD(Ser112)(7E11) (No.9296), BAD No.9292), BAD control proteins (No.9293), cytochrome c (No.4272) were from Cell Signaling Technology; anti-phospho-Bcl2 (Ser70), clone CT7 (No.05-843) was from Upstate; Bcl-2 (Ab-1) (No.OP60) was from Calbiochem, anti-Bcl-2, clone-100 (B-3170), monoclonal anti-actin, clone AC-40 (A-4700), monoclonal anti-β-tubulin, clone TUB 2.1 (T-4026) were from Sigma. Immunoprecipitation reagent Protein A/G Plus-Agarose (sc-2003) was from Santa Cruz Biotechnology. Secondary antibodies, goat anti-mouse IgG (No.115-035-146) and goat anti-rabbit IgG (No.111-035-144) were from Jackson ImmunoResearch Laboratories. Protein molecular weight SeeBlue®Pre-Stained Standard (LC5625) was from Invitrogen. Western Lightning Chemiluminescence reagent (NEL101) was from PerkinElmer Life Sciences and Kodak™ BioMax® Light-1 autoradiography films (No.8194540) were from Amersham Biosciences.

Cell Culture

HUVEC were seeded in 2%-gelatine-coated T-75 flask at a density of 1-3 x10^4 cells/cm^2 in MCDB131 medium containing L-glutamine 2mM, penicillin-G (100 U/ml), streptomycin sulfate (100 μg/ml), amphotericin B (Fungizone) (0.25 μg/ml), supplemented with 15% fetal bovine serum (FBS), endothelial cell growth factor (20 μg/ml), heparin (7.5 U/ml) and hydrocortisone (0.15 μg/ml). Cells were re-fed daily and subcultured at 90 % of confluency. For experimental studies cells were switched to red phenol-free (RPF) (546), steroid hormone and growth factors deprived media (547). Cells were trypsinized (0.05% Trypsin/EDTA, RPF) and seeded at a density of 1-3 x10^4 cells/cm^2 on 60 mm plates for flow cytometry, or on 6-well plates for protein analysis in transition medium consisting of M199-RPF, L-glutamine (2mM), antibiotics (1%), MgSO4 (2%), sodium selenite (22 nM), FBS (15%) and growth factors. When cells reached 90% confluency (~ 48 hr), cell culture media was switched to steroid- and growth factor-depleted RPF media containing 15% of charcoal stripped heat-inactivated serum (CSS-HI) with addition of transferrin (0.5 μg/ml) for 12-24 hours (referred to as incomplete medium-MIC). Before the stimulation for cell cycle analysis cells were synchronized for 4-6 hours by starvation in M199-RPF, glutamine, antibiotics, MgSO4, sodium selenite, transferrin and 0.02% of bovine serum albumin (BSA). For cell treatments BSA in this media was replaced by 10% of CSS-HI.

Cell cycle analysis was performed by flow cytometry (fluorescence-activated cell sorting, FACS analysis) (548). Single cell suspension was prepared from floating cells in the media that were combined with the cells released by trypsinization (0.05%-

Trypsin/EDTA, RPF) and pelleted (250x g for 5 min at room temperature). Cells were washed twice with Dulbecco's PBS (DPBS) lacking Ca2+ and Mg 2+. The final pellet was resuspended in 0.5 ml DPBS and fixed in 4 ml of ice-cold 70-% ethanol. For the propidium iodide (PI) staining, the ethanol-fixed cell suspension was spun and washed again. The staining solution consisted of 0.1%-v/v Triton X-100, EDTA (0.5 mM), PI (0.05 mg/ml) and RNAse A (0.05 mg/ml) in DPBS at pH 7.4.

DNA content was measured using FACScan™ or FACScalibur. Sheath fluid was isotonic saline solution. Red fluorescence of PI stained DNA was visualized as fluorescence 3 (FL3) on linear scale at medium or high flow rate corresponding to 50-100 events/s. Data were acquired using CellQuestPro™ program. Unstained cells were used as blank and provided a standard for gating out DNA-free cell debris. The threshold was set at 30-50 of the FL3 channel to further exclude cell debris. The red fluorescence peak representing G0/G1 was consistently positioned on the channel 200 in the linear scale of the FL3 channel. At least 10,000 total events for each sample were analyzed. The initial instrument setting was maintained during the data acquisition for all samples and samples were prepared in duplicates from independent culture dishes.

The acquired data were analyzed using FCSExpress DeNovo, Summit v4 DakoCytomation and WinMDI 2.8 software. Cell debris and doublets were gated out on scattered plot. The apoptotic cells were quantified as hypodiploid cell population.

Western Blot

At the end of stimulation, the conditioning media containing floating cells was collected into 15-ml tubes and pelleted at 250x g for 5 min. Adherent cells in wells were washed once with ice cold DPBS, lysed in 75 µl of lysis buffer (containing in mM: Hepes 50, sodium chloride 150, sodium fluoride 100, sodium pyrophosphate 10, EDTA 5, 1% Triton X-100, pH 7.5, protease inhibitors aprotinin 5 µg/ml, leupeptin 2 µg/ml, pepstatin 1 µg/ml, activated sodium orthovanadate 1 mM) scraped and added to the pellet from floating cells. Lysates were passed ~10x through an insulin syringe and microcentrifuged at 13,000 rpm (18,000x g) for 10 min at 4°C and in some experiments at 3,000 rpm (700x g). Protein in supernatant was determined by the method of Bradford and equal concentrations of proteins used for electrophoresis.

Anti-phospho-Bcl-2 (20 µg) was immunoprecipitated with 0.2 mg/ml of lysate protein and with 50 µl of protein A/G Plus agarose overnight at 4°C. The pelleted beads were washed 3x with ice cold PBS and spun and then added to SDS-sample buffer (10x) and samples were boiled for 5 min. The entire reaction mixture was resolved by 12%-SDS-PAGE.

Separated proteins were transferred to polyvinylidene fluoride (PVDF) membrane, blocked with 5%-milk-TBST (Tris base 10 mM, NaCl 100 mM, Tween 0.1%, pH 7.5%) shaking for 1 hour at room temperature and probed with primary antibodies overnight at 4°C and probed with peroxidase-conjugated secondary antibodies. Blots were incubated with LumiLight reagent and visualized on films. Autoradiographs were scanned and intensity of bands was quantified by measuring optical density in AlphaEaseFC software. Results are based on biological replicates which varied from 6 – 12.

Statistical Analysis

Statistical analysis was performed using One-way RM ANOVA and post hoc analysis by Student-Newman-Keuls test or Paired t-test (SigmaStat software, SPSS Inc.). $P < 0.05$ was considered significant. Error bars are plus minus SEM.

Oxidation Extent of Oxidized LDL

Human oxidized LDL was obtained commercially from Intracel company where it was isolated from fresh human plasma by sequential ultracentrifugation. Oxidized LDL was prepared by the company by incubating LDL with CuSO4 also by company and sold as mg of protein/ml. Purchased, oxLDL was dialyzed in phosphate buffer saline through a dialysis membrane Spectra/Por (6 – 8,000 MWCO) and sterile filtered (0.45 μm). We measured lipid hydroperoxides in the oxLDL spectrophotometrically at 560 nm using PeroxiDetectTM kit and quantified with a standard curve. The mean values expressed in nmol of hydroperoxides per mg of protein (Bradford) were 1059.8 ± 84.4 nmol/mg for oxLDL vs 79.7 ± 54.1 nmol/mg in native LDL (*Fig. 1 A*). We ascertained these results by relative electrophoretic mobility assay (REM) (549) with LIPO electrophoresis kit (No.655910) on Paragon® electrophoresis system from Beckman Coulter. REM indicated that the mobility of oxLDL on agarose gel was 3.9 ± 0.3 fold higher compared with native LDL (*Fig. 1 B*). Fresh oxLDL was used within 7 –10 days in experiment.

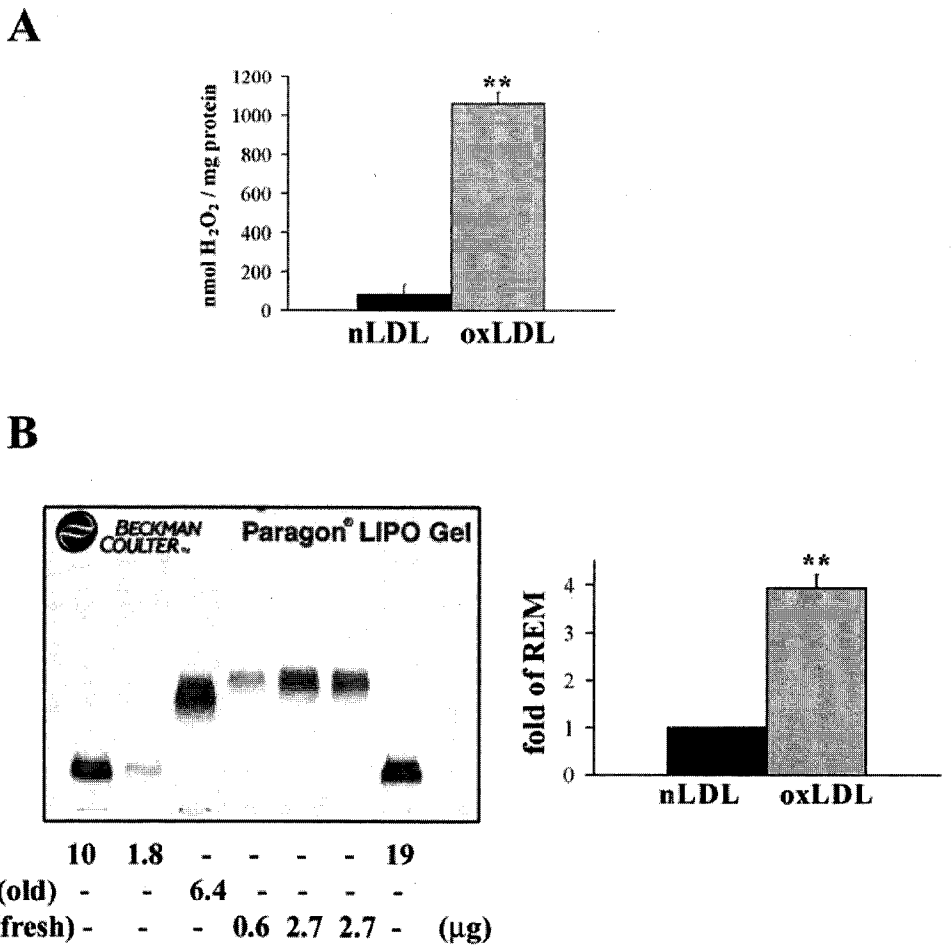

Figure III-1. The extent of oxidation of oxLDL throughout the experiments.

(**A**) The level of hydroperoxides in oxidized lipoprotein (oxLDL) compared to unmodified native lipoprotein (nLDL). (**B**). An example of lipoprotein electrophoresis on agarose gel. REM is measured as the distance in milimetres from the bands formed by nLDL in lane 1,2 or 7. (µg) refers to the protein amount of lipoprotein per lane and shows that REM depends on the degree of oxidation of modified lipids rather than on loaded quantity. oxLDL (old) is a few weeks old product, oxLDL (fresh) is the product measured within 5 days after preparation. The graph to the right shows the means of 6 independent experiments,

** $P < 0.001$.

III.4. RESULTS

The Induction of Apoptosis by oxLDL

We first established the dose-response of oxLDL induced apoptosis by flow cytometry and cleaved caspase-3 expression (*Fig. 2 A, B*). Incubation of HUVEC for 24-hour increased apoptosis beginning with 50 μg/ml and peaked at 75 μg/ml.

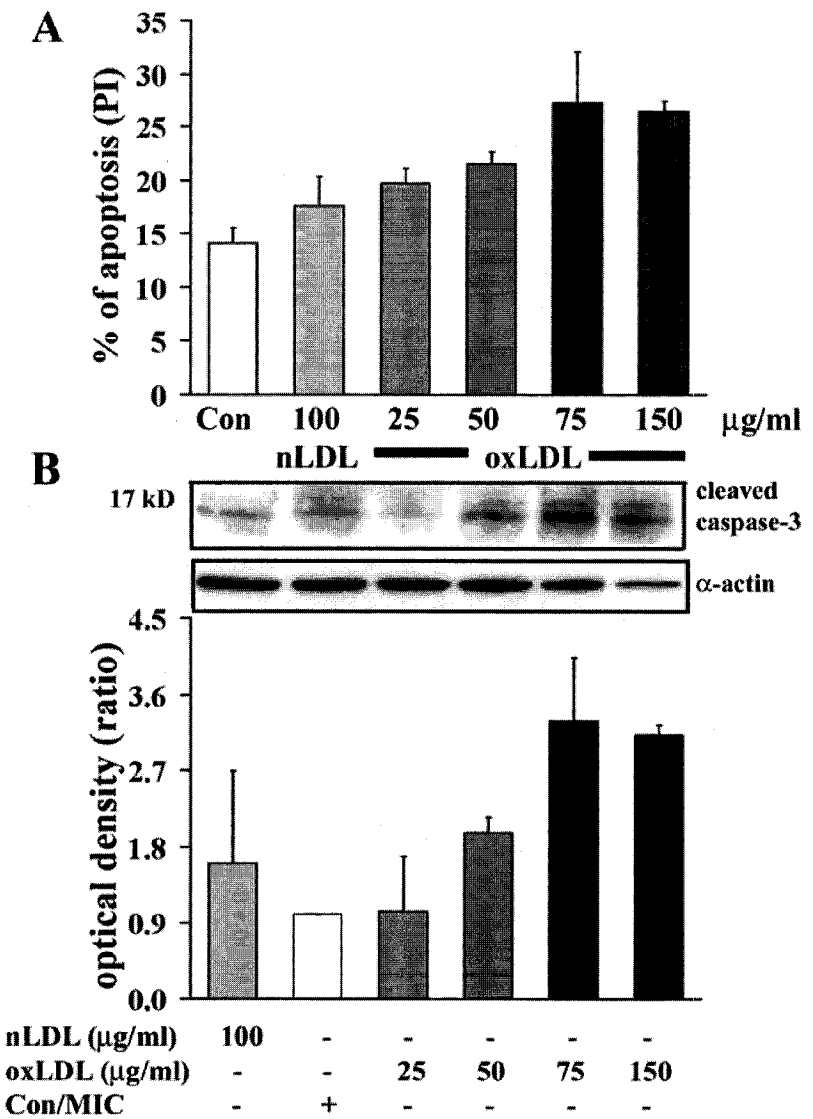

Figure III-2. Dose-response effect of oxLDL on apoptosis induction in HUVEC.

(**A**) Flow cytometry of HUVEC treated for 24 hours in red phenol-free, growth factor and steroid hormone-depleted medium alone (Con), nLDL (native LDL 100 µg/ml) or increasing concentrations of oxLDL (oxidized LDL 25, 50, 75, 150 µg/ml). (**B**) Immunoblotting for cleaved caspase-3 expression at 17 kD (top row). Stripped membranes were reprobed for α-actin (bottom row) to verify protein loading. Bar graph of optical density (ratio of control) of cleaved caspase-3 (n = 2).

Effect of E2 on Apoptosis Induced by TNFα and oxLDL at 24 hours

The hypodiploid peak in the sub-G0/G1 phase of cell cycle, which indicates apoptosis was increased by both TNFα and oxLDL after 24 hours of treatment. E2 reduced TNFα induced apoptosis from 12.9 % ± 1.48 to 9.9 % ± 1.13 ($P<0.05$) (*Fig. 3 A*) and that of oxLDL from 12.3 % ± 0.81 to 9.5 % ± 0.94 ($P<0.01$) (*Fig. 3 B*).

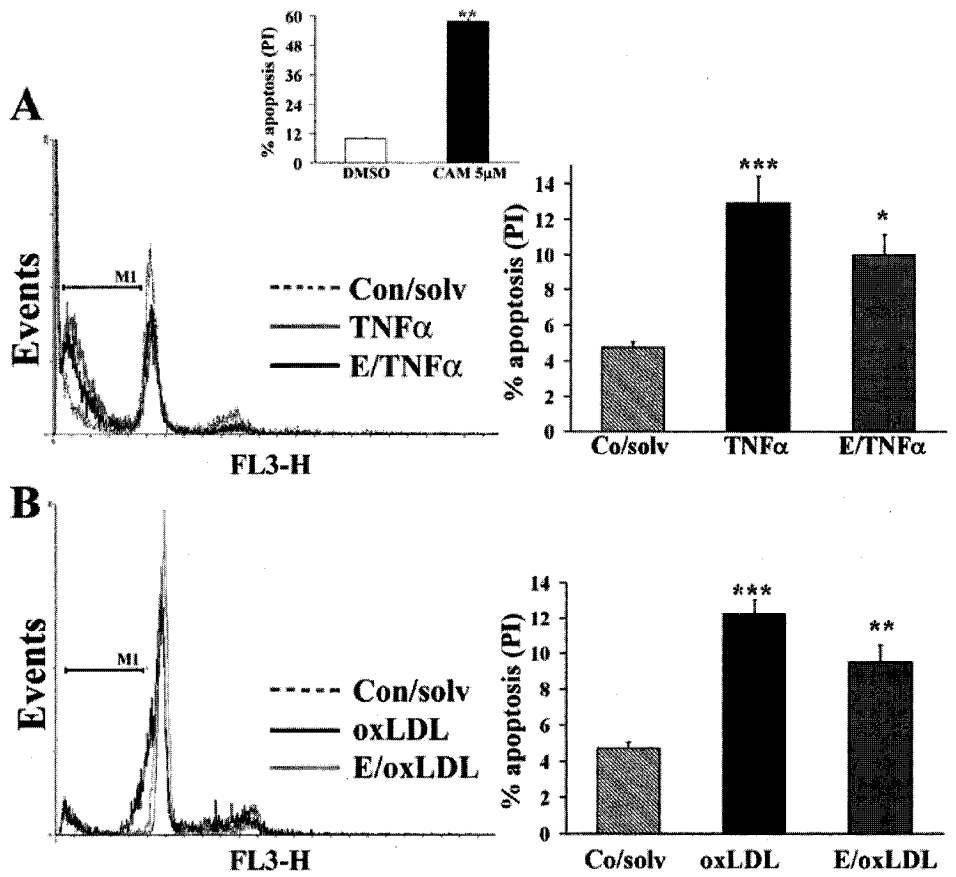

Figure III-3. Flow cytometric analysis of effects of E2 on TNFα and oxLDL induced apoptosis.

Examples of flow cytometry of PI stained cells. Apoptosis was quantified from fluorescence signals below G1 phase peak gated (marker M1) on forward scatter (FL3-H) and expressed as percentage of the total number of events. (**A**) 24-hour exposure to solvent control (0.0005% v/v) (ethanol) (*thin dotted line*), TNFα 20 ng/ml (*light full line*) or E2 20 nM combined with TNFα (*black line*). Bar graph (right) shows summary of a minimum of 6 independent experiments. Apoptosis of HUVEC stimulated with camptothecin (CAM) 5 μM or its solvent (DMSO) served as an internal control of the experiment (*inset*). TNFα increased apoptosis; addition of E2 reduced this increase. (**B**) 24-hour exposure to solvent control (*thin dotted line*), oxLDL (75 μg/ml) (*black line*) or E2 plus oxLDL addition (*light full line*). The bar graph (right) shows the mean of a minimum 12 independent experiments. (*P< 0.05, ** P< 0.01, *** P< 0.001).

Estradiol in HUVEC Diminished the Cleaved Caspase-3 Expression at 24 hours

After 24-hr treatment with oxLDL the active form of caspase-3 at 17 kDa was increased (*Fig. 4 A*). Addition of E2 to oxLDL treated cells decreased caspase activation. The band at ~ 30 kDa is procaspase and confirms equal expression of the full protein. Treatment of HUVEC for 24 hours with TNFα also increased cleavage of caspase-3 and E2 reduced this TNFα induction of active caspase-3 (*Fig. 4 B*).

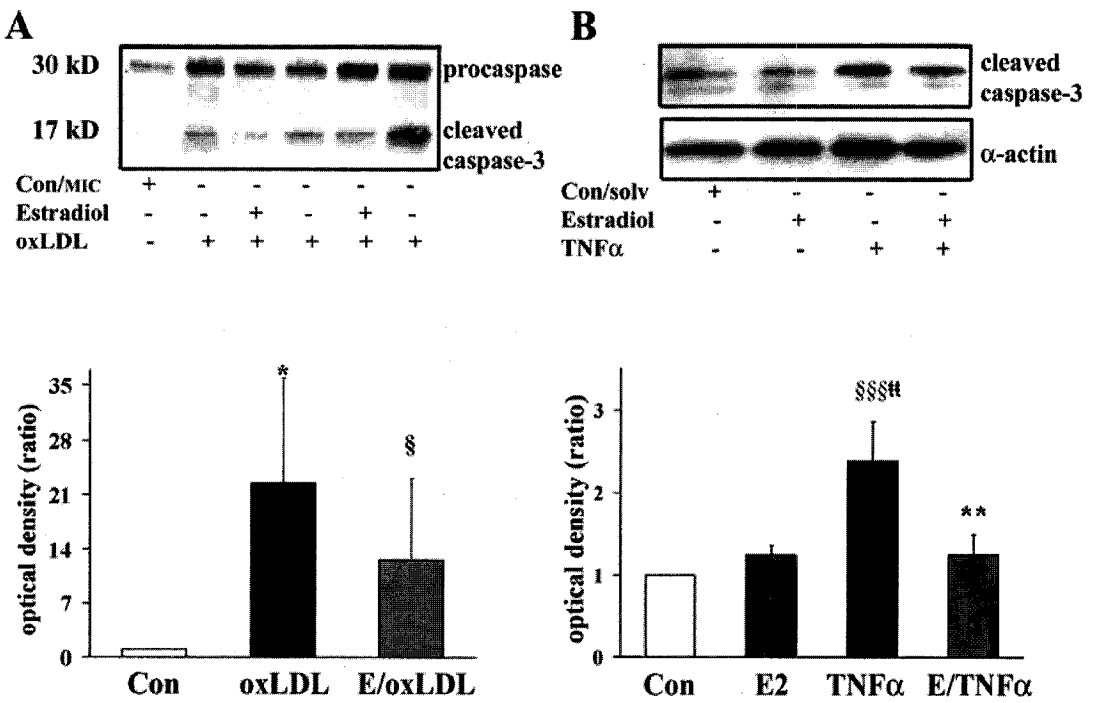

Figure III-4. Attenuation by E2 of cleaved caspase-3 expression in HUVEC in response to treatment with oxLDL or TNFα for 24 hours.

An example of cells treated with oxLDL is shown on the left and TNFα on the right. Summary data are shown in the bar graphs below. (**A**) Caspase-3 activity was increased by oxLDL and the induction was reduced by E2. (* P< 0.05 Con/MIC vs. oxLDL, § P< 0.05 oxLDL vs. E/oxLDL)(n=10-20). (**B**) Caspase-3 activity was increased by TNFα and reduced by co-treatment with E2. One-way RM ANOVA was significant as follows (n=12):
§§§ P< 0.001 TNFα vs Con, ǂǂ P< 0.01 TNFα vs. E2, ** P< 0.01 E/TNFα vs. TNFα.

Effect of E2 on The Induction of Apoptosis with TNFα and oxLDL at 6 hours

At 6 hours TNFα (*Fig. 5 A*) increased apoptotic cells to 9.1% and E2 did not prevent the increase. OxLDL did not induce apoptosis at 6 hours (*Fig. 5 B*) (n = 2). TNFα (*Fig. 5 C, top row*) also induced a large increase of caspase-3 cleavage at 6 hours and this too was not altered by E2. As we observed with flow cytometry, 6-hour treatment of HUVEC with oxLDL did not induce activation of cleaved caspase-3 and there was no change with E2 (*Fig. 5 C*).

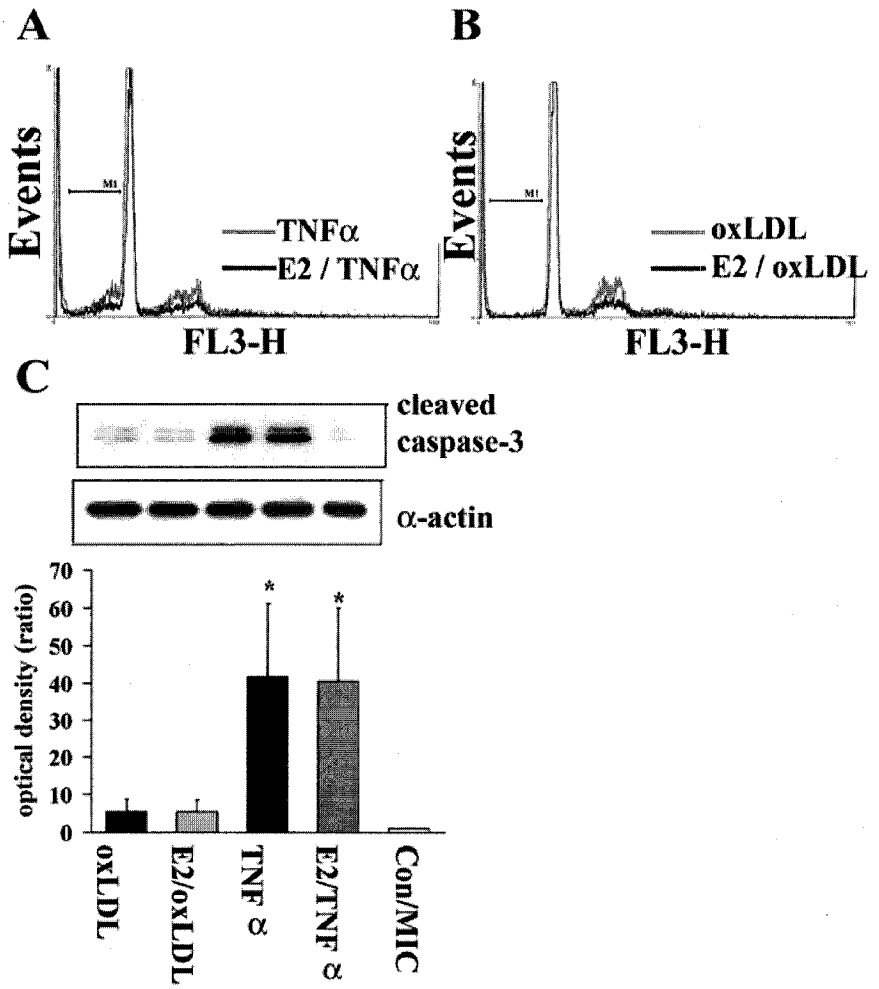

Figure III-5. The effect of E2 on apoptosis induction by oxLDL and TNFα at 6 hr in HUVEC.

(**A**) Shows an example of flow cytometry of cells treated with TNFα (20 ng/ml) with or without E2 and (**B**) or oxLDL (75 μg/ml) with or without E2. (**C**) Shows a sample western analysis of caspase-3 activity with oxLDL or TNFα (blot). The graph represents the average of 3-6 independent experiments * $P < 0.05$.

Involvement of Antiapoptotic Bcl-2 Proteins in TNFα and oxLDL Induced Apoptosis in HUVEC

We investigated the role of Bcl proteins in the apoptotic response at 6 hours and 24 hours. TNFα, oxLDL, and E2 had no effect on the expression of Bcl-2 or Bcl-xL at 6 hours (*Fig. 6 D*). TNFα also had no effect on expression of Bcl-2 or Bcl-xL at 24 hours (*Fig. 6 A*). On the other hand, at 24 hours oxLDL increased the expression of Bcl-xL but not Bcl-2 (*Fig. 6 B*). At 24 hours, E2 increased the expression of Bcl-xL and this persisted in oxLDL treated cells (*Fig. 6 C*) ($P<0.05$) but not in TNFα treated cells. E2 also had no effect on Bcl-2 phosphorylation (data shown as *supplementary material in Fig. 9*).

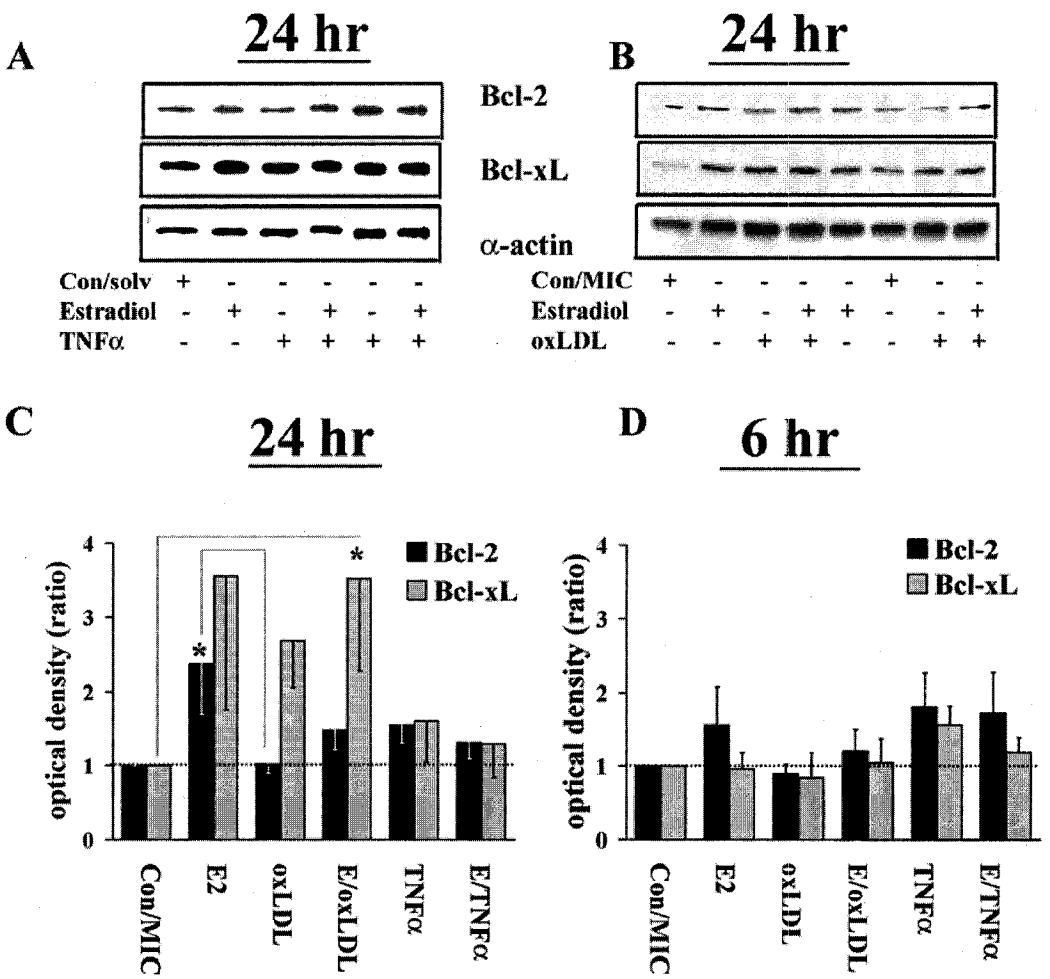

Figure III-6. Expression of Bcl-2 and Bcl-xL in response to TNFα and oxLDL for 6 and 24 hr with or without E2.

An example of western blots of Bcl-2, Bcl-xL and α-actin as a control for protein loading of HUVEC induced with TNFα (**A**) or oxLDL (**B**) in 24 hr-treated cells. As shown in the summary bar graph for 24 hr (**C**) E2 increased Bcl-2 and Bcl-xL and TNFα prevented this rise. OxLDL increased Bcl-xL expression but not that of Bcl-2. E2 did not alter this further. * $P < 0.05$ (n = 8-12). (**D**) At 6 hr there were no changes in Bcl-2 or Bcl-xL protein expression with any of the treatments (n = 5).

Estradiol Induces BAD Phosphorylation

Treatment of HUVEC with E2 produced a time dependent increase in BAD phosphorylation particularly at 30 minutes and 1 hour (*Fig. 7 A, top row*). Expression of total BAD did not change (*middle row*). After 24 hours of treatment of HUVEC with E2 BAD phosphorylation increased (*Fig. 7 B, top row*), but so did total BAD (*middle row*) so that the ratio of phosphorylated to non-phosphorylated actually decreased (*Fig. 7 B, graph*). The ratio was not altered when E2 was added.

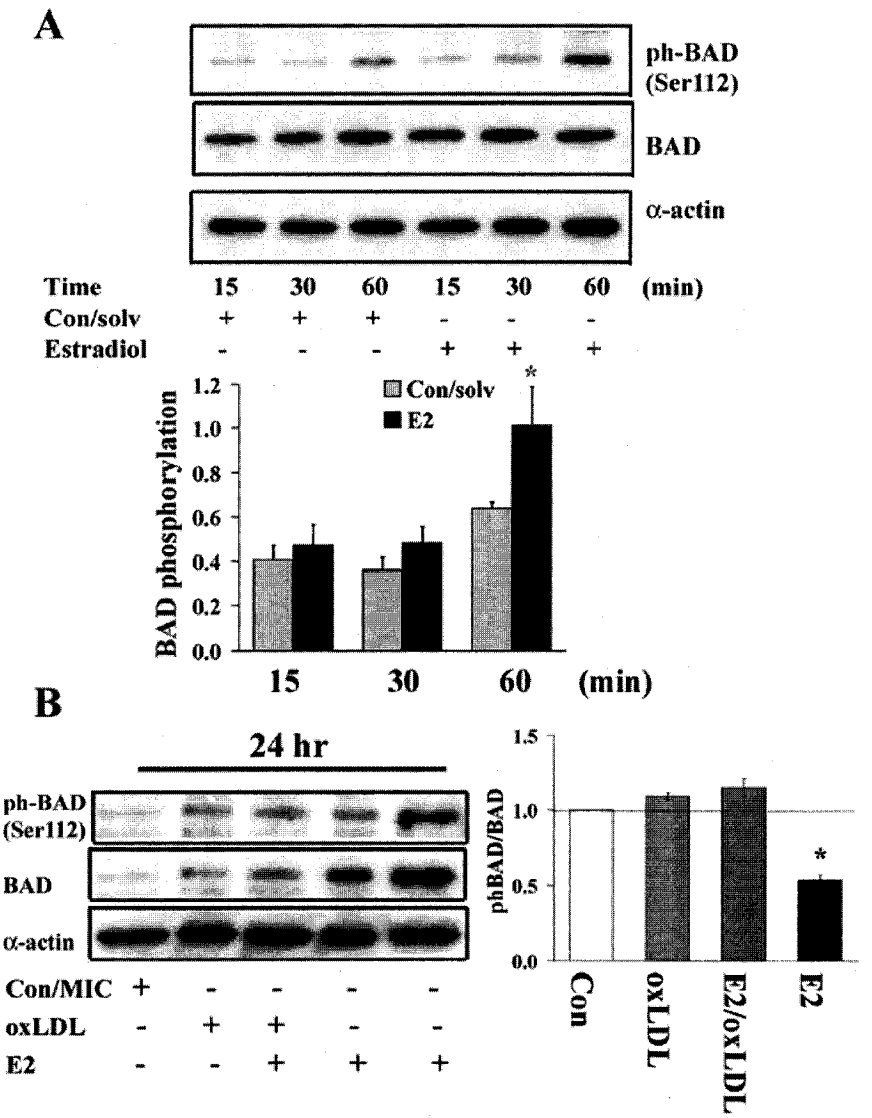

Figure III-7. Western blot of BAD phosphorylation at serine 112.

(**A**) Shows the early time course of stimulation with E2 20 nM or solvent (ethanol 0.0005% v/v final concentration) and (**B**) shows results at 24 hr. Blots were re-probed with total anti-BAD and α-actin to verify protein loading (n = 3). E2 increased phosphorylation of BAD at 60 min (bar graph in center) * $P < 0.01$ by paired t-test. 24 hr of treatment with E2 increased total BAD as well as phosphorylated BAD so that the ratio of phospho-BAD/BAD total decreased.

Involvement of Phosphatidylinositol-3-kinase (PI3K) in E2 Suppression of Endothelial Cell Death Triggered by oxLDL

The photomicrographs of HUVEC in *Figure 8* demonstrate the morphological changes that occurred in cells exposed for 24 hours to various conditions. After 24 hours, HUVEC cultured in complete medium (MC) displayed the typical cobblestone shape, which is a characteristic of confluent endothelial cells in early passages. In contrast, there were many floating cells (black spots on the micrograph) even at time zero when cells were cultured in medium that was stripped of growth factors (MIC). By 24 hours, there were empty spaces between cells and white spots that indicate non-viable or dying cells. The addition of oxLDL to the cell culture greatly reduced the density of the monolayer and this reduction was somewhat less when E2 was added to the cell culture indicating that E2 protected the cells from the negative effect of oxLDL. When Wortmannin (W) was added many more cells were detached and cells were elongated and sparse. This suggests that the PI(3)K/AKT pathway contributes to the survival of these cells.

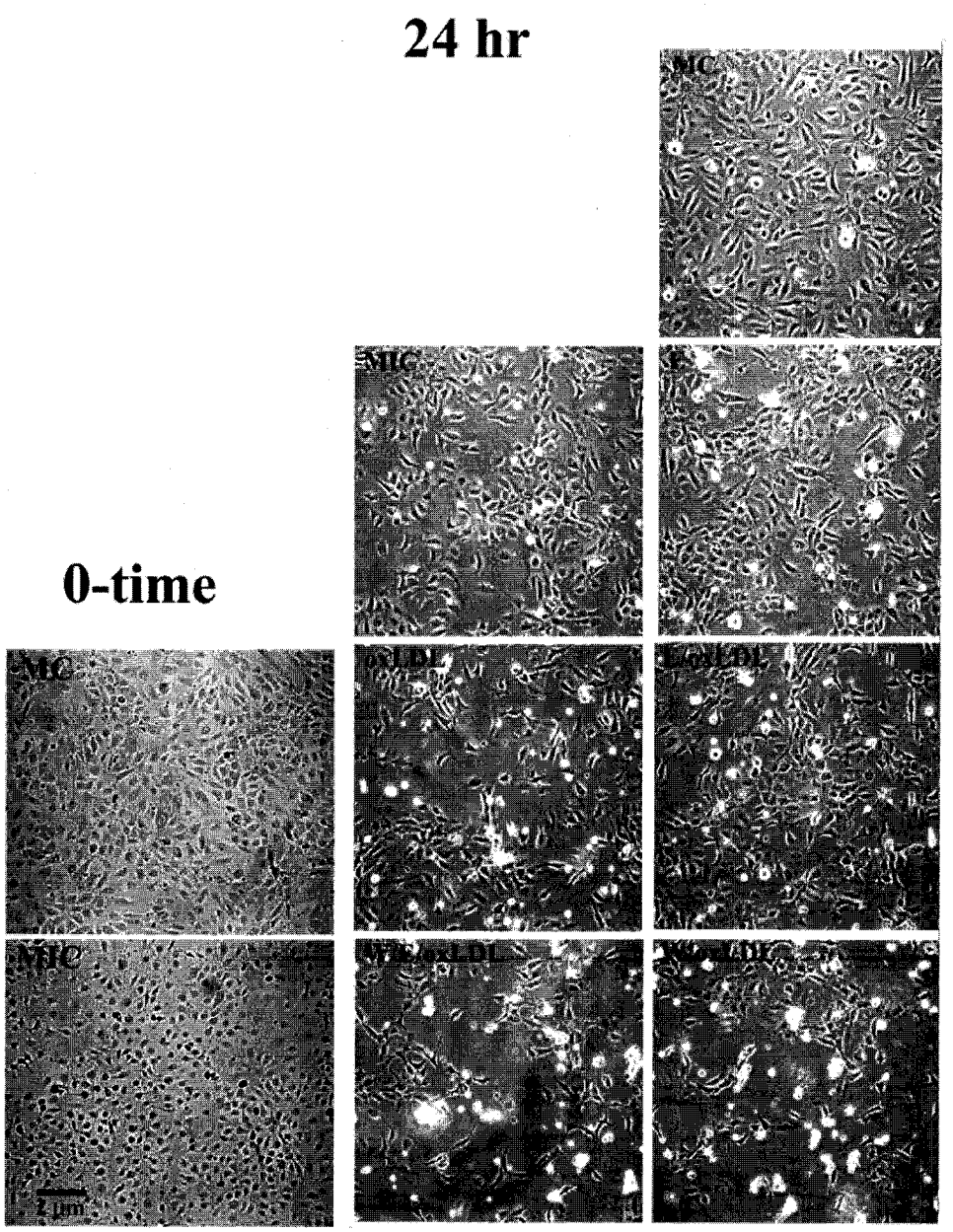

Figure III-8. Photomicrographs of cells in cultures

Time 0 indicates cells in complete media (MC) and steroid depleted incomplete media (MIC). Cultures are also shown at the end of the 24-hour treatments in MC or MIC alone or with E2, oxLDL, Wortmannin (W) or in combinations. Captions were taken on microscope Olympus IX70 in the brightfield, magnification 100 x. Scale bar = 2 mm.

E2 did not influence the Bcl-2 phosphorylation

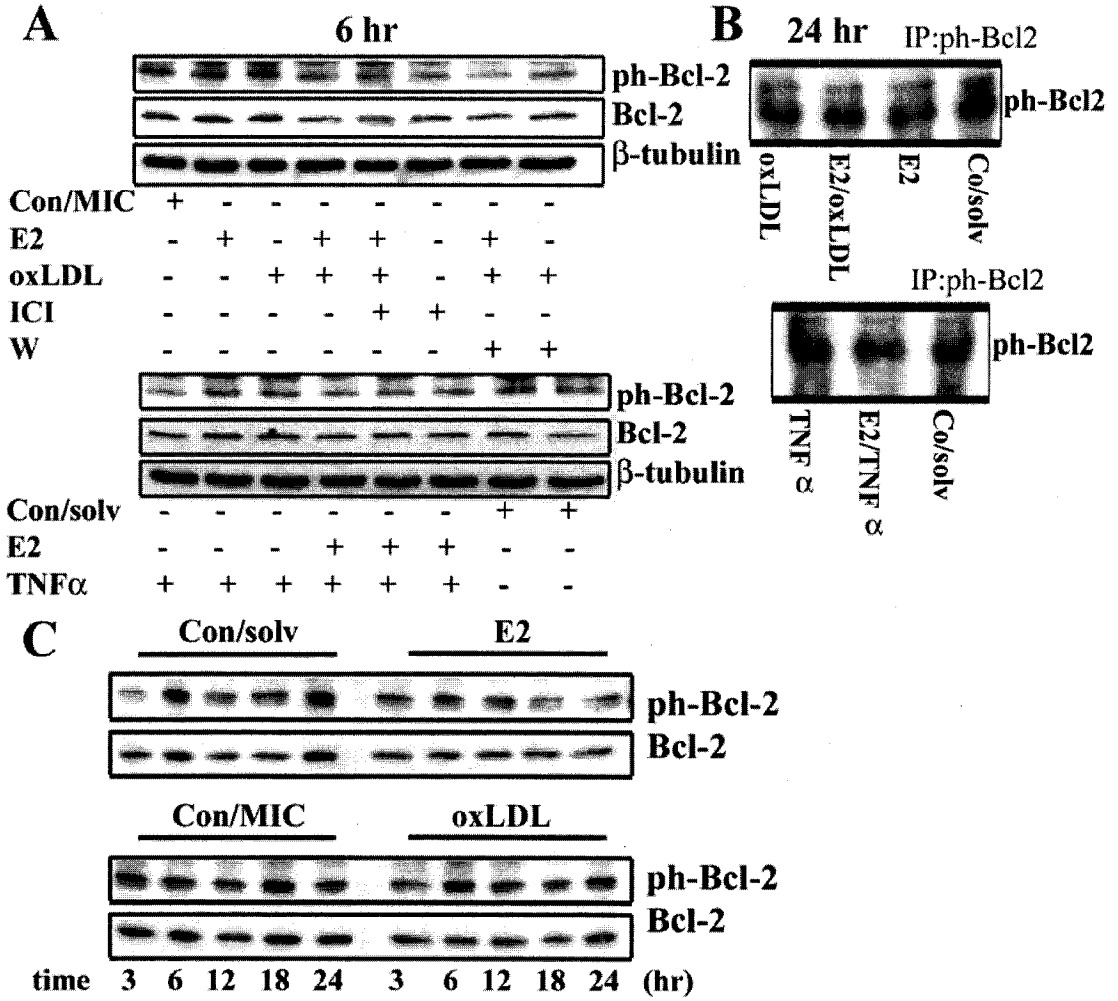

Figure III-9, supplementary. Phosphorylation of Bcl-2 by E2 treatments with oxLDL and TNFα in time.

(**A**) Effect at 6-hour of E2 on phospho-Bcl-2 expression in oxLDL (upper blots) stimulated cells or in combination with ICI 182,780 1 μM or with Wortmannin (W) 1 μM or in TNFα (lower blots) treated. Blots were reprobed for total Bcl-2 and β-tubulin. Activity of phospho-Bcl-2 (**B**) in immunoprecipitates treated 24-hr as indicated (top and bottom row). Time-course (**C**) with E2, oxLDL and respective controls on Bcl-2 phosphorylation. In relation in total Bcl-2 there were no changes in phospho-Bcl-2 expression by any of the treatments except Wortmannin, which decreased phosphorylation.

III.5. Discussion

Apoptosis of cells in atherosclerotic lesions is thought to contribute to plaque destabilization and cardiovascular events (494;544;550). Since estrogens have anti-apoptotic properties in endothelial cells (394), cancer cells (128;404), as well as in vivo (496), estrogen could be vasculoprotective. We thus studied the potential for estrogen to reduce apoptosis in endothelial cells treated with TNFα and oxLDL, two known proapoptotic substances that play a role in the progression of atherosclerosis (551). The effect of estrogen on TNFα induced apoptosis has been previously studied (394), but we included TNFα as a reference for the effect of oxLDL. We confirmed that estrogen reduces TNFα induced apoptosis in HUVEC treated with E2 for 24 hours and found that estrogen also reduces oxLDL induced apoptosis to a similar degree. However, the reduction of apoptosis produced by both agonists was not large so that the net in vivo effect would depend on the balance between the prevention of apoptosis and the strength of the pro-apoptotic signal. Furthermore, estrogen did not alter early apoptosis induced by TNFα and only reduced the apoptosis seen at 24 hours. Our results suggest that the action of estrogen requires the induction of anti-apoptotic proteins or only acts upon factors that act later in the apoptotic process. This would also mean that the endothelium would have to be functional and able to produce anti-apoptotic factors, which might not be the case in advanced atherosclerotic lesions.

Technical limitations

Before discussing our findings, some potential limitations needed to be reviewed. Perhaps the most important issue is the use of HUVEC to examine the antiapoptotic effects of estrogens. We chose HUVEC because they are a primary human cell type, they have been widely used to study apoptosis (500;501;510;552-555), they are readily available and they come from a large conductance vessel, which is the type of vessel in which atherosclerosis begins, rather than a microvascular vessel. HUVEC are primary cells and quickly loose their phenotype in culture so we used only early passage cells. Because the phenotype of HUVEC is variable we used cells pooled from a number of donors to minimize the variation. We studied cells at near confluence because this is closer to the state that endothelial cells are in vivo. However we did not study cells at actual confluence because the proapoptotic effect of TNFα is known to be cell-cycle dependent (499). The state of confluence is directly related to cell density, which is an important determinant of the effect of oxLDL. The cytotoxic effect of oxLDL is much greater at low densities (556) and results in greater necrosis than apoptosis (557). This may explain some of the variability in the assessment of apoptosis with oxLDL in the literature.

A major problem in studying the effect of estrogen on apoptosis is that it is necessary to strip steroid hormones and other growth factors from the serum with use of charcoal-dextran (547) so that the specific action of estrogen can be identified. However serum deprivation triggers apoptosis and charcoal stripping of serum increased the amount of apoptosis above that seen with full serum. Charcoal-treatment also occasionally resulted in vacuolization and senescence of cells, which suggests some

necrosis instead of apoptosis. This could have been due to activation of complement in charcoal-stripped serum because of removal of endogenous inhibitors. In support of this, heat inactivation of charcoal-stripped serum reduced this problem but the increase in baseline apoptosis still limited our ability to quantitate the anti-apoptotic effects of estrogen by raising the signal-to-noise ratio. However, the rates of apoptosis induced by TNFα and oxLDL were still much lower than that induced by the topoisomerase inhibitor, camptothecin indicating that a significant portion of the untreated cells were viable and able to undergo apoptosis.

We studied oxLDL because of its pathological importance, but oxLDL is not an easy substance to use experimentally. The apoptotic effect of oxLDL is dependent upon its concentration and the degree of oxidation (558). To establish an appropriate dose we assessed the dose-response relationship and found that maximum apoptosis at 24 hours as determined by both flow cytometry and cleaved caspase-3 occurred at 75 μg/ml of oxLDL. Based on measurement of lipid hydroperoxides and REM as an assessment of the extent of oxidation, the potency of our oxLDL was at the higher end of values reported by others (500;559;560). Despite our attempts to standardize the potency of oxLDL, there likely still was variability in the extent of oxidation. This is believed to occur partly because of an unequal number of copper binding sites among donor LDL (561). Finally another technical problem that can lead to variability is that oxLDL can adsorb estrogens and potentially reduce their uptake by the endothelial cells (51).

Spyridopoulos et al previously showed that estrogen treatment reduces TNFα induced apoptosis in endothelial cells (394). TNFα induced apoptosis in their study was much higher than we observed, perhaps because cells were studied at 70% confluence

and the dose of TNFα was greater. Estrogen has also been shown to reduce endothelial apoptosis induced by serum deprivation (504).

OxLDL plays an important role in atherosclerosis (506;562) and indeed oxLDL has been shown to induce endothelial apoptosis (496;500;510;552;553;555;563-566). We found that oxLDL increased apoptosis in a dose-dependent manner with a peak effect at 75 μg/ml. We also found that TNFα induced apoptosis after only 6 hours of exposure, but apoptosis with oxLDL was only evident after 24 hours at which time the magnitude of apoptosis as assessed by flow-cytometry and caspase-3 cleavage was very similar to that we observed with TNFα. In most other studies, oxLDL induced apoptosis has only been examined after 20 hours (500;564;566;567). In one study in which oxLDL induced apoptosis was examined at 10 hours, the extent of apoptosis was only about one third of that seen at 24 hours (553). In another study, oxLDL was found to induce apoptosis after 12 hours and in contrast to what we observed, apoptosis did not seem to be related to activation of caspase-3 (568). The authors attributed the action of oxLDL to be due to activation of calpain and subsequent proteolysis of BID to its pro-apoptotic form. The effect of estrogen on oxLDL induced apoptosis has not been previously studied. We found that estrogen reduced oxLDL induced apoptosis and the magnitude of that reduction was very similar to the reduction of TNFα induced apoptosis by estrogen.

A number of mechanisms have been proposed to explain the apoptotic effect of oxLDL. One mechanism is related to Fas (CD95/Apo-1), a type 1 transmembrane protein belonging to the TNFα receptor family, and Fas ligand (FasL) system. Ligand activation of Fas results in oligomerization and recruitment of Fas-associated death domain (FADD) and pro-FLICE (FADD-homologous ICE-like protease) and the cleavage of pro-FLICE.

Active FLICE then triggers the caspase cascade and apoptosis. Sata and Walsh showed that endothelial cells derived from mice that do not have Fas or FasL have more apoptosis in response to oxLDL than wild type mice (554). It at first is surprising that activation of this system would trigger apoptosis because endothelial cells have both Fas and FasL in place and are normally resistant to activation of Fas (554). However, in a number of cells that have both Fas and FasL, including endothelial cells, this process is kept in check by FLICE inhibitory protein (FLIP) and Sata and Walsh showed that oxLDL induced apoptosis occurs by down-regulating FLIP (555). The loss of FLIP was demonstrated at 20 hours suggesting that the process takes time and could explain the more delayed occurrence of oxLDL induced apoptosis compared to that seen with TNFα. This could possibly be due to differences in the death domain of TNFα compared to FAS or even to bypass of the death domain pathway through the 80 kDa TNF receptor (569). This Fas mechanism could be further increased by induction of Fas by oxLDL (564). FLIP expression is thought to be dependent upon the PI(3)K/AKT pathway (570) which is activated by estrogen (136;138). Thus estrogen activation of AKT could provide an explanation for the reduction of oxLDL induced apoptosis.

Induction of apoptosis in endothelial cells has also been shown to be related to production of reactive oxygen species (ROS) for transfection of cells with catalase (565) or addition of superoxide dismutase (510) reduced the degree of apoptosis in isolated cells. Catalase also reduced apoptosis due to oxLDL in situ carotid arteries of rats (496). A possible source for increased ROS has been suggested to be NADPH oxidase activation by oxLDL of (571). It was proposed that the increase in ROS would change the redox balance which then could affect the activity of Bcl proteins as discussed below. We

previously found that estrogen treatment decreases the activation of NAD(P)H oxidase by reducing the migration of cytoplasmic $p47^{phox}$ to the cell membrane where it is needed for full activity of the enzyme (339)(see *Chapter II* and *Appendix 1*) and thus this process, too, could contribute to the antiapoptotic effect of estrogen in cells treated with oxLDL.

Activation of death domains by receptor-mediated mechanisms directly increases apoptosis, but apoptosis can also be regulated by factors that inhibit apoptosis produced by mitochondrial mechanisms. The Bcl family of proteins is important for this function. They can be regulated by transcription or phosphorylation. For example, taxol or UV radiation increase apoptosis by phosphorylating and inactivating the antiapoptotic proteins Bcl-2 and Bcl-xL and this is prevented by estrogen. We and others (554) found that neither TNFα nor oxLDL altered expression of Bcl-2 and Bcl-xL, although one group found that oxLDL decreased bcl-2 expression in endothelial cells (564). On the other hand, estrogen treatment increased Bcl-2 and Bcl-xL expression, which could have contributed to the antiapoptotic effect of estrogen. In support of this finding, Pike et al found that estrogen increased Bcl-xL expression in cultured hippocampal neurons and identified an estrogen response element in the bcl-x gene (572). Importantly the increase in expression of Bcl-2 and Bcl-xL only occurred at 24 hours and was not present at 6 hours, which is consistent with the later protective effect of estrogen that we observed. We also observed that oxLDL increased expression of Bcl-xL at 24 hours but not 6 hours. In contrast, others have found that estrogen withdrawal did not decrease expression of bcl-2 or bcl-xl (573).

We found that oxLDL itself increased Bcl-xL expression. This was also observed by Hundal et al in macrophages (574) and they argued that the increase in Bcl-xL could lead

to increased atherosclerosis because it means there would be less apoptosis of macrophages invading the vascular wall (574). However, an increase in Bcl-xL in endothelial cells would be protective. Estrogen in our study resulted in a further increase in Bcl-xL expression above the increase induced by oxLDL.

We also examined phosphorylation of the proapoptotic protein, BAD, which would decrease its apoptotic activity (575). Estrogen treatment increased BAD phosphorylation at 6 hours. Similarly, estrogen induced phosphorylation of BAD in breast cancer cells which resulted in inhibition of apoptosis induced by TNFα and serum deprivation (404). This action of estrogen occurred through a Ras/PI(3)K dependent mechanism and PI(3)K is also activated by estrogen in endothelial cells (138). The result at 24 hours was more complicated for estrogen increased total BAD as well as phospho-BAD such that the ratio of phospho-BAD to total BAD actually went down. The final implications of this for apoptosis are not clear, but might mean that prolonged estrogen exposure could have harmful effects.

In conclusion, estrogen inhibits apoptosis induced by TNFα and oxLDL in HUVEC. However, the inhibition is not complete and is delayed in that estrogen did not inhibit the early induction of apoptosis produced by TNFα. The delayed action of estrogen on inhibition of apoptosis suggests the need for induction of factors such as the increase in Bcl proteins, Bcl-2 and Bcl-xL, that we observed, and possible effects on inhibitors of the death domain pathway such as FLIP. This inhibition of apoptosis in endothelial cells would potentially be vasculoprotective, however inhibition of estrogen in other cell types such as macrophages could promote atherosclerosis. Furthermore, the antiapoptotic effect of estrogen on endothelial cells can only occur if the cells can manufacture anti-apoptotic

molecules in response to estrogen. These contradictory effects of estrogen are consistent with contradictory clinical results with hormone replacement therapy in post-menopausal women.

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Bridge between the second and the third manuscript.

Estrogen is known to have both positive and negative effects on the vasculature. The prevention of apoptosis is important for endothelial health. We showed that E2 reduced apoptosis induced by TNFα and oxLDL. The antiapoptotic effects were primarily through a reduction of caspase-3 activation and an increase in BAD phosphorylation. The process required the activation of the PI(3)K/AKT survival pathway.

Inhibition of EC apoptosis leading to EC survival is thought to be an essential issue during angiogenesis. However, the effects of E2 on the vasculature become problematic when inflammation and established atherosclerosis are present. The proangiogenic effects of E2 may be particularly important in a situation of advanced atherosclerotic plaques because they increase neovascularization, thus contributing to the progression of atherosclerotic disease. The role of increased angiogenesis in atherogenesis has been recently emphasized and linked to the neovessel formation in carcinogenesis (517), which strengthens our hypothesis that whereas in vasculature without major atherosclerotic disease E2 helps maintain a quiescent endothelium, in chronic inflammation the proangiogenic effects of E2 worsen the cardiovascular pathology.

IV. Estrogen influences angiogenesis by interacting with TNFα

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IV.1. Abstract

Objective – Estrogen's place in women's health remains controversial. Observational studies have shown protection from cardiovascular disease (CVD) whereas randomized trials failed to find a benefit and even suggested harm. We hypothesized that this paradox may be explained by estrogen's potent pro-angiogenic property, which could increase neovascularization and destabilize advanced atherosclerotic plaques, especially in the presence of chronic inflammation which is often seen in older women. To explore this possibility we assessed angiogenesis in human umbilical vein endothelial cells (HUVEC), and examined the interaction of 17-β estradiol (E2) and the inflammatory cytokine TNFα.

Methods – To identify angiogenesis, we measured migration and proliferation. Early passage of HUVEC was grown to confluence. Cells were grown in red phenol free media with charcoal stripped serum (10% CSS) to remove estrogen. A scratch was made across the plate (wound assay). Migration across the wound was observed and quantified over the next 12-48 hours. We also assessed proliferation with BrDU. Cells were treated with media alone, TNFα at 0.3, 1 or 20 ng/ml, E2 at 20 nM or the combination of E2 and TNFα. We used real time PCR to measure changes in expression of angiogenesis genes angiopoeitin-2 (Ang-2), VEGF-A and C and IL-8.

Results – We found 67.6 ± 6.4 % of healed area after 24 hours with E2 compared to 37.5 ± 1.25 % in the control. Large dose TNFα (20ng/ml) almost completely inhibited healing and the addition of E2 preserved some healing. Low-dose TNFα (0.3 ng/ml) had no effect whereas 1.0 ng/ml moderately increased migration to 43.8 ± 1.6 %. The addition of E2 to

0.3 ng/ml of TNFα increased migration to 50.2 ± 2.5 % and the addition of E2 to 1.0 ng/ml of TNFα increased it to 54.2 ± 2.6 %, but the final value was less than that of E2 alone. E2 treatment time-dependently increased migration in wounded HUVEC that were pretreated with TNFα, that is up to 51.9 ± 4.1 % at 24 hours compared to an untreated control (28.3 ± 1.0 %). In contrast, HUVEC pretreated with E2 and subsequently treated with TNFα did not show progress of migration at 24 hours (29.6 ± 1.7 %) compared to the control. E2 induced a small but significant increase in DNA synthesis of HUVEC after 24 hours of treatment. 0.3 and 1.0 ng/ml of TNFα suppressed EC proliferation. Both concentrations of TNFα (0.3 and 1.0 ng/ml) increased the expression of VEGF-C to 2.8 ± 0.1 and 2.5 ± 0.2 fold of the control respectively. E2 partially prevented this effect to 2.3 ± 0.2 and to 1.9 ± 0.1 fold in TNFα 0.3 and 1.0 ng/ml respectively. The IL-8 expression increased to 32.8 ± 1.2 and 42.7 ± 3.6 respectively. The addition of E2 decreased it to 24.2 ± 3.4 and 33.5 ± 2.2 fold of the control respectively.

Conclusion – E2 increases angiogenesis induced by TNFα, which potentially could worsen the stability of complex atherosclerotic plaques and lead to cardiovascular event.

IV.2. Introduction

The role of E2 in women's health remains contradictory, particularly in the area of cardiovascular disease (CVD). Population studies indicated that premenopausal women have less CVD than males of parallel age. Menopause precludes these differences and CVD occurred at the same rate in postmenopausal women as in the same aged men (1;2;19;484;520;521;542). Women on homone replacement therapy (HRT) have less CVD than women who were not (83;484). Studies on primates (193;194) and rodents (80;82;191;576-579) showed that E2 protects against atherosclerotic disease and increases healing of vascular wounds.

Mechanisms to explain beneficial effects of E2 have also been identified (20;111;580;581). Increased nitric oxide (NO) production by activation of endothelial nitric oxide synthase (eNOS) mediated by the PI(3)K/AKT pathway is one of the most important mechanism(146). Also, E2 can suppress apoptosis in endothelial cells (ECs) (143;394) and decrease expression of inflammatory proteins (71).

Angiogenesis plays a crucial role in wound healing and in the perpetuation of chronic inflammatory diseases (582). E2 is also proangiogenic as suggested by neovascularization that physiologically occurs in the uterus during the menstrual cycle and pregnancy (465). E2 induced angiogenesis has been proposed to explain the better healing of vascular injury in animals with intact estrogen receptors (ERs) than in animals without E2 (82). E2 enhances fibroblast growth factor (FGF)-2 induced angiogenesis in an *in vivo* model (192). In OVX mice, the angiogenic response to FGF-2 is reduced, and E2-replacement restores the angiogenic response to FGF-2 (192). In addition, it has been

shown that disruption of functional ER gene reduces FGF-2-induced angiogenesis by exogenous E2 in OVX mice (583). E2 increases EC attachment to extracellular matrix proteins (192). This effect of E2 is rapid, mediated by integrins and by increased tyrosine phosphorylation (584). Thus, E2 regulates multiple signaling pathways between growth factors (585;586), integrins (587) and proteases (588), key participants in new vessel formation (463).

E2 may have positive and negative consequences on cardiovascular system. E2 could protect women from CVD by accelerating repair of injured endothelium and restoring an anti-atherogenic state (28). However, E2 induced angiogenesis contributes to increased incidence of breast cancer in women on HRT (2). Neovascularization is common around plaques and these new vessels can lead to plaque instability and rupture (29). Furthermore, diseased areas of the vascular wall may have been denuded of endothelium so that E2 could not increase eNOS activity, one of its primary beneficial effects and the raw surfaces would be more susceptible to E2 prothrombotic effects (589).

TNFα seems to be an important factor in the inflammation and subsequently into the progression of CVD in postmenopausal women (551). TNFα is increased in hypertension (590) and diabetes (591;592), two important risk factors for coronary artery disease (CAD). It has been shown to decrease expression of nitric oxide (NO) and increase superoxide (O^{2-}) production by NAD(P)H oxidase (593).

E2 has been shown to modulate serum TNFα. Serum TNFα was higher in OVX, E2-deficient rats than E2-treated or intact rats. Furthermore, eNOS expression was increased and expression of the component of NAD(P)H complex were decreased (341). Increased

TNFα associated with E2-deficiency impaired functional recovery from ischemia-reperfusion and restoring E2 improved outcomes (594).

In quiescent endothelium, E2 promotes its maintenance and minor repairs of injured endothelium (28). However, when E2 is given in the presence of active inflammatory molecules such as TNFα, the proangiogenic and prothrombotic effects could potentiate each other. Furthermore, TNFα could negate the benefits of E2 on increased NO production. Thus, we hypothesize that E2 will modulate the response of the endothelium to TNFα.

IV.3. Materials and Methods

Cryopreserved human umbilical vein endothelial cells (HUVEC) were obtained from GlycoTech (No.41-001, Gaithersburg, MD) or Cambrex Bio Science (CC-2519, Walkersville, MD). Cell culture media and supplements were obtained from Invitrogen unless otherwise stated. Subsequent components were obtained as follows: charcoal stripped delipidated fetal bovine serum (C-1696), gelatine type B solution (G-1393), magnesium sulfate solution (M-3409), sodium selenite (S-9133), human apo-transferrin (T-5391), bovine serum albumin (A-8806), TNFα (T-6674), endothelial cell growth supplement (E-2759) from Sigma, endothelial mitogen (ECGS) from Biomedical Technologies (BT-203, Stoughton, MA), estradiol in powder from Steraloids (E0950-000, Newport RI). Cell proliferation ELISA, BrdU colorimetric kit from Roche (11647229001), Trypan Blue Stain 0.4% from Invitrogen (15250061).

Primary antibodies for western blotting were from Cell Signaling Technology: anti-phospho-PAK1 (Ser199/204) (No.2605), anti-phospho-PAK1 (Ser144) (No.2606), anti-

phospho-PAK1 (Thr423) (No.2601), anti-PAK1 (No.2602). Secondary antibody, a goat anti-rabbit IgG (No.111-035-144) was from Jackson ImmunoResearch Laboratories. Protein molecular weight SeeBlue®Pre-Stained Standard (LC5625) was from Invitrogen. Western Lightning Chemiluminescence reagent (NEL101) was from PerkinElmer Life Sciences and Kodak™ BioMax® Light-1 autoradiography films (No.8194540) were from Amersham Biosciences.

Cell Culture

HUVEC were seeded in the 2%-gelatine-coated T-75 flask at the density $1.5x10^4$ cells/cm^2 in MCDB131 medium containing L-glutamin 2mM, penicillin-G (100 U/ml), streptomycin sulfate (100 μg/ml), amphotericin B (Fungizone) (0.25 μg/ml), supplemented with 15% fetal bovine serum (FBS), 20 μg/ml endothelial cell growth factor (ECGF), 7.5 U/ml heparin and 0.15 μg/ml hydrocortisone. Cells were refed daily and subcultured at 90 % of confluency in the ratio 1:6 that corresponded to population doubling (PDL) 2.5 – 3. PDL was calculated using the following formula: PDL = ($\log_{10}$F – $\lg_{10}$I) / 0.301 (F indicates number of cells at the end of the passage; I is initial number of cells when seeded)(501).

For experimental studies cells, in few steps, were switched to red phenol-free (RPF) (546), steroid hormone and growth factors deprived media (547). First, cells were trypsinized (0.05% Trypsin/EDTA- RPF) and seeded at the density $1.5x10^4$ cells/cm^2 onto 6-well plates for wound injury assay, direct cell counting and for real-time PCR, or in 96-well plates for BrDU proliferation assay. Cells were cultured in transition medium

consisting of M199-RPF, L-glutamine (2mM), antibiotics (1%), supplemented with 2% $MgSO_4$, 22 nM sodium selenite, 15% FBS and growth factors. When cells reached 90% confluency (~ 48 hr), cell culture media was switched to steroid-depleted RPF media containing 5% of charcoal stripped fetal bovine serum heat-inactivated (CSS-HI) with addition of transferrin 0.5 μg/ml, ECGF 20 μg/ml, and heparin 7.5 U/ml, but not hydrocortisone, for 12 hours (referred to as incomplete medium-MIC). For cell treatments M199-RPF with glutamine, antibiotics, $MgSO_4$, sodium selenite, transferrin and 7% of CSS-HI was used. In some experiments, cells were withdrawn from CSS-HI and starved for 4-6 hours in 0.02% bovine serum albumin (BSA) only before stimulation. We used cells until the 4^{th} passage.

Cell protocol for wound injury (migration) assay

Nearly confluent cell monolayers ready for stimulation were wounded in a cross-shaped pattern with a sterile 200 μl pipette tip. The media and dislodged cells were aspirated and plates were replenished with conditioning media only and served as a control (MIC) or were replenished with media containing E2 20 nM, TNFα 0.3, 1, 10 or 20 ng/ml, or a combination of these. For the *pretreatment* experiment, HUVEC were pretreated in medium alone (MIC), with E2 20 nM or with TNFα 1.0 ng/ml for 4 hours. Cells were then wounded and replenished with media containing treatments (MIC alone, E2 or TNFα). Wound healing at different time points (0, 6, 12, 24, 36, 48 hours) was photographed in the same scratched area localized to the right of the cross-shaped scratch with a 40x objective of the microscope Olympus IX70 and quantified by measuring the wounded area with Image Pro-Plus software. Results were reported as the percentage of

wound healing using the equation: % wound healing = [1-(wound area at $T_{12\ hrs}$ / wound area at T_0)] x 100, where T_0 is the time immediately following wounding.

Proliferation assay

EC proliferation (or DNA synthesis) was performed by 5-bromo-2'-deoxyuridine (BrdU) incorporation by using a colorimetric cell proliferation (ELISA) kit as per the manufacturer's instructions. Cells were plated on 96-well plate at density 5 x 10^4 in RPF media M199. Nearly confluent cells were switched to conditioning media depleted of growth factors and steroids, containing 15% of CSS-HI, 10 μM BrdU labeling reagent and the ligands of interest for 24 hours. Cells were fixed, then anti-BrdU peroxidase conjugated antibody was added. The immune complexes were detected by the subsequent substrate reaction and data were acquired on an ELISA microplate reader (SpectraMax M2 spectrophotometer through SoftMax Pro software). The absorbance was measured at dual wavelength using the 370 nm filter and the 492 nm wavelength filter, which served as the reference.

Direct counting of enzymatically dissociated cells with Trypan blue exclusion

Cells were cultured in 6-well plates and treated for 24 hours in RPF conditioning media deprived from growth factors and steroid hormones. At the end of the stimulation, conditioning medium was aspirated and cells were washed once with Dulbecco's phosphate buffered saline. Cells were trypsinized with 0.05% Trypsin/EDTA – RPF, pelleted at 2000 rpm for 5 min at room temperature and resuspended in 0.5 ml of basal

medium. Cells were mixed with Trypan blue at the ratio 2:1 and counted by a hemocytometer. Final count was expressed as a number of viable cells x 10^5 / 1 ml.

Real-Time PCR protocol

Cell protocol: Nearly confluent HUVEC cultured in 6-well plates in RPF conditioning medium E2-depleted, were scratched, medium was replenished by conditioning medium with 10% CSS-HI, containing ligands of interest. Cells were harvested and RNA was extracted after 6, 12 and 24 hours of stimulation. At indicated time points cell cultures were also photographed with objectives 40x and 100x of an Olympus IX70 microscope.

RNA extraction: Total RNA was extracted from cultured cells using the GenElute™ Mammalian Total RNA Kit from Sigma (RTN70). The extractions were carried out according to the manufacturer's instructions. The concentration and the purity of RNA were determined by measuring the absorbance at 260 nm and the A_{260}/A_{280} ratio in a spectrophotometer SpectraMax-M2. 2 μg of total RNA in 20 μl of final reaction volume were reverse transcribed into cDNA using the Superscript II RT Gibco/Life (18064-014) from Invitrogen.

Real-time PCR: Gene relative quantitation was performed by Real-Time PCR using FAM™ fluorescent TaqMan probes (IL-8 No.HS00174103_m1 306045 and VEGF-A No.HS00173626_m1 317167). Reactions were performed using TaqMan®Universal Master Mix (Applied Biosystems, Inc.) and 100 ng of cDNA in a final volume of 20 μl. The ABI 7500 Real Time PCR System was used with the following protocol: 95°C for 10

min., followed by 55 cycles at 95°C for 15 sec. and at 60°C for 1 min. An alternate set of primers was designed using the dye SYBR®Green: VEGF-C forward (F) primer: TGTACAAGTGTCAGCTAAGG, VEGF-C reverse (R) primer: CCACATCTATACACACCTCC giving a product of 183 bp, Ang-2 primer F: ATAAGCAGCATCAGCCAACCA, Ang-2 primer R: CATTCCGTTCAAGTTGGAAGGA giving a product of 136 bp, AngL-4 primer F: CCACTTGGACCAGGATCAC, AngL-4 primer R: CGGAAGTACTGGCCGTTGAG giving a product of 115 bp and GAPDH (endogenous probe F: AAGAAGGTGGTGAAGCAGGCG, probe R: ACCAGGAAATGAGCTTGACAA) giving a product of 166 bp. The specificity of the Real PCR product with SYBR Green I Dye was verified by dissociation curves for every gene. For each specific product correspond only one peak. Gene expression analysis using these primers was performed on ABI 7500 Real Time PCR System under the following conditions: 95°C for 10 min., followed by 50 cycles at 95°C for 15 sec., at 57°C for 30 sec., and at 72°C for 33 sec. Experiments were carried in duplicates.

Data analysis

The level of product in proportion to the level of starting point was assessed by Ct (threshold cycle number). The Ct values were calculated from the mid-point of the exponential phase of amplification. The Ct, defined as the cycle at which PCR amplification reaches a significant value above the calculated baseline fluorescence, was given as the mean value. The relative expression of each mRNA was calculated by the ΔCt method.

Western blot analysis

At the end of stimulation, adherent cells in wells were washed once with ice cold DPBS, lysed in 75 µl of lysis buffer (containing in mM: Hepes 50, sodium chloride 150, sodium fluoride 100, sodium pyrophosphate 10, EDTA 5, 1% Triton X-100, pH 7.5, protease inhibitors aprotinin 5 µg/ml, leupeptin 2 µg/ml, pepstatin 1 µg/ml, activated sodium orthovanadate 1 mM) and scraped. Lysates were passed ~5x through an insulin syringe and microcentrifuged at 12,000 rpm (18,000x g) for 10 min at 4°C. Protein in supernatant was determined by the method of Bradford and equal concentrations of proteins used for electrophoresis. To the protein samples was added the SDS-sample buffer (10x) and samples were boiled for 5 min. The entire reaction mixture was resolved by 8%-SDS-PAGE. Separated proteins were transferred to polyvinylidene fluoride (PVDF) membrane, blocked with 5%-milk-TBST (Tris base 10 mM, NaCl 100 mM, Tween 0.1%, pH 7.5%) shaking for 1 hour at room temperature and probed with primary antibodies overnight at 4°C and probed with peroxidase-conjugated secondary antibodies. Blots were incubated with LumiLight reagent and visualized on films.

IV.4. RESULTS

Wound injury / Migration with high dose of TNFα

We have performed a wound injury / migration assay with a high dose of TNFα (20 ng/ml) in HUVEC. At 12 hours, E2 alone increased migration (expressed as percentage

of wound healing) up to 42.6 ± 1.4 % whereas TNFα-treated cells migrated to cover only 21.5 ± 0.8 % of the wounded area (*Fig. 1 A*). A plateau of migration was maintained at 24, 36 and 48 hours with E2, but with TNFα this process degraded. In contrast, the addition of E2 to TNFα partially restored migration. Photomicrographs (*Fig. 1 B*) illustrated the time course of wound repair. While wound repopulation progressed with time in E2 treated EC until practically complete healing at the end of the stimulation, TNFα did not demonstrate any wound healing. Furthermore, with time the density of TNFα-treated cells surrounding the wound diminished. The addition of E2 to TNFα reduced the wounded area over the time and occasional cells could be found migrating into the healing area.

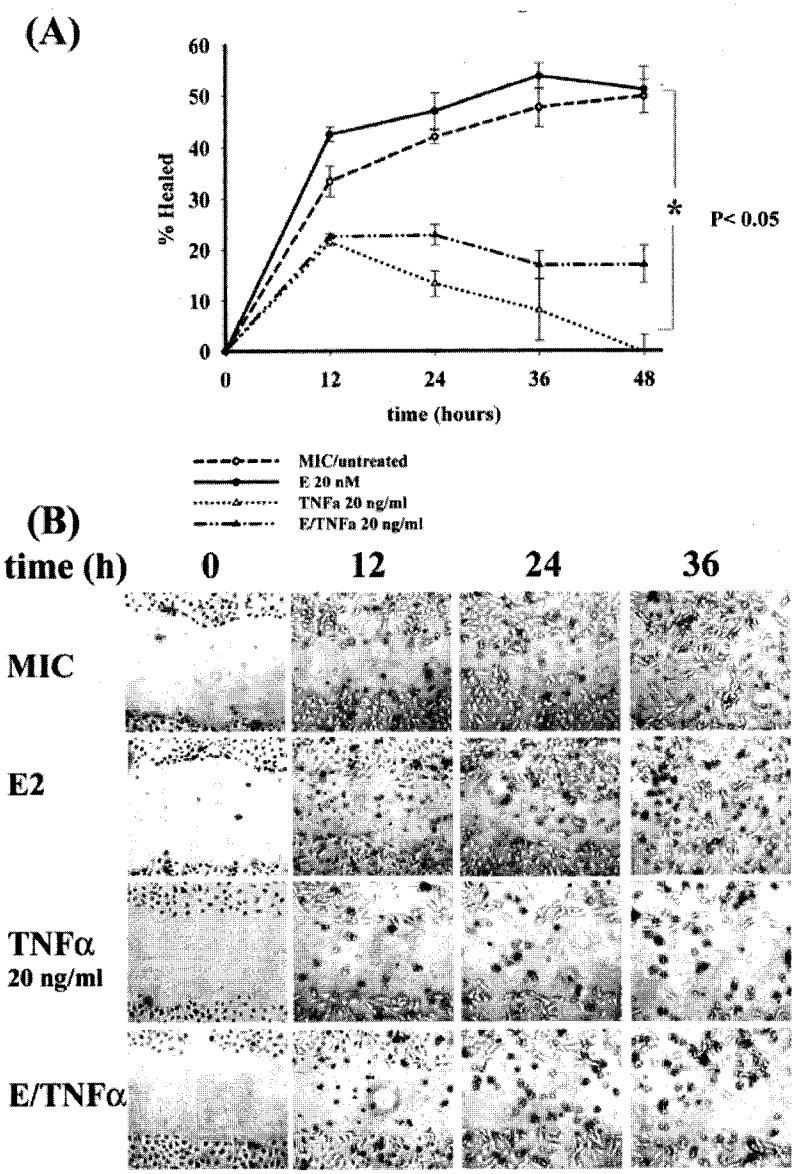


Figure IV-1. Time-course of migration of HUVEC in wound assay with high dose of TNFα.

(**A**). A graphical representation of wound healing. There was a small increase in migration with E2 compared to untreated control (MIC) beginning at 12 hours. TNFα (20 ng/ml) did not increase migration and E2 partially restored it and maintained it over the time. (**B**). Photomicrographs illustrating the sequence of wound healing in HUVEC treated in conditioning media alone, with E2, TNFα or in combination at indicated time points (40x magnification). (n = 3).

Wound injury / Migration was dependent on TNFα-concentration

E2 alone significantly increased migration of HUVECs (*Fig. 2 A*). At 24 hours, the migration after wound injury almost doubled to that of the untreated control (MIC). We found 67.6 ± 6.4 % of healed area with E2 compared to 37.5 ± 1.25 % in the control. 0.3 ng/ml of TNFα had no effect and 1.0 ng/ml moderately increased migration to 43.8 ± 1.6 %. The addition of E2 to 0.3 ng/ml of TNFα increased migration to 50.2 ± 2.5 % and the addition of E2 to 1.0 ng/ml of TNFα increased it to 54.2 ± 2.6 %, but the final value was less than that of E2 alone. The examples of migration after wound injury captured on photomicrographs (*Fig. 2 B*) confirmed the robust wound repair with E2 alone or when added to TNFα 1.0 ng/ml. However, after 24 hours the scratch edges were still very even around the denuded area in cells treated with TNFα 0.3 ng/ml alone or with the addition of E2.

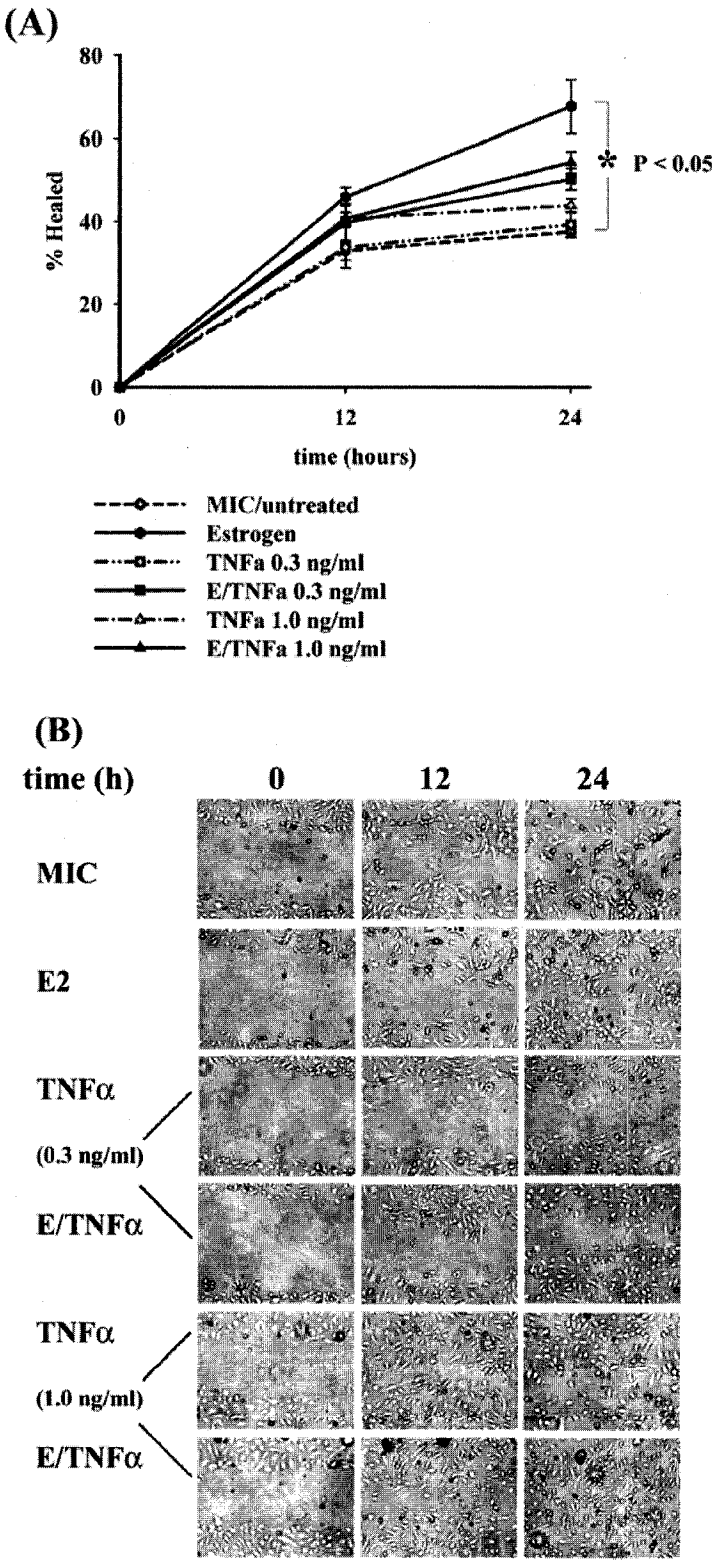
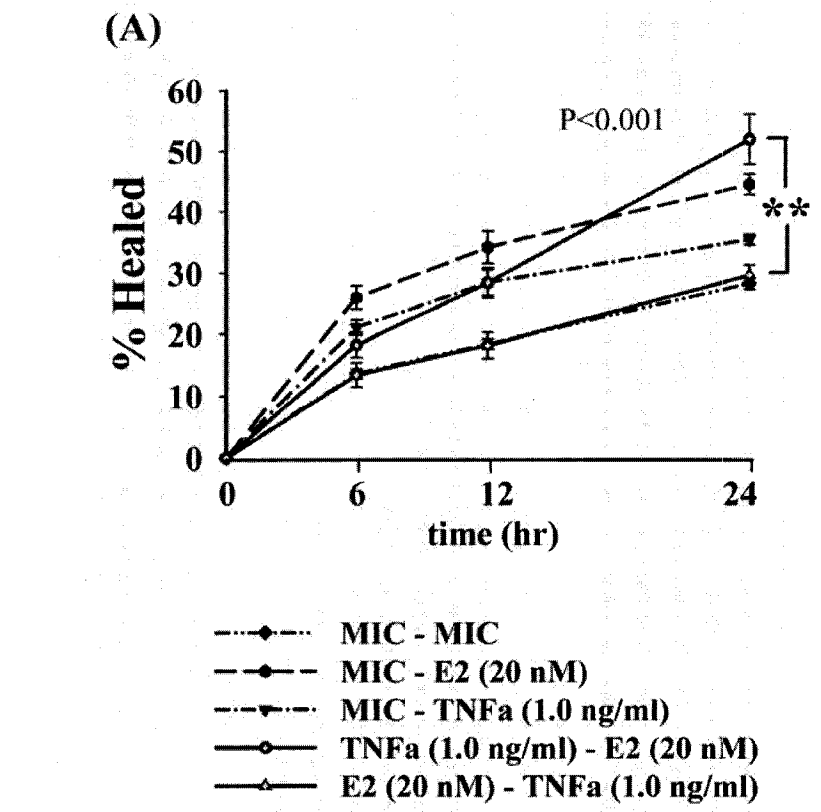


Figure IV-2. Migration of HUVEC in wound assay with lower doses of TNFα.

(**A**). E2 alone significantly increased migration. 1.0 but not 0.3 ng/ml of TNFα increased migration and the addition of E2 to both doses of TNFα increased migration. (**B**). Photomicrographs of HUVEC wounded at the beginning of stimulations (0 h). Wounds were sequentially photographed at 0, 12 and 24 hours (40x magnification). (n = 3)

Estrogen significantly increased migration in TNFα pretreated HUVEC

E2 treatment time-dependently increased migration in wounded HUVEC that were pretreated with TNFα (TNFα - E) for 4 hours, that is up to 51.9 ± 4.1 % at 24 hours (*Fig. 3 A*). HUVEC pretreated in medium without any ligands (MIC) and subsequently treated with E2 (MIC – E2) demonstrated 44.5 ± 1.7% of migration; on the other hand, TNFα treatment (MIC – TNFα) caused 35.4 ± 0.9% of HUVEC migration into the wounded area. In contrast, HUVEC pretreated for 4 hours with E2 and subsequently treated with TNFα (E2 – TNFα) did not show progress of migration at 24 hours compared to untreated cells before or after the scratch wound (MIC - MIC), 29.6 ± 1.7 % and 28.3 ± 1.0 % respectively. Morphological changes (*Fig. 3 B*) after wounding showed already after 12 hours that HUVEC pretreated (Pre-Tx) with TNFα and treated (Tx) with E2 spread intensively from boundaries to the wounded area. At 24 hours the original wound is almost healed and unrecognizable from the rest of the monolayer. There is only very little migration from the border zone to the denuded area in cells preatreted with E2 and subsequently treated with TNFα.



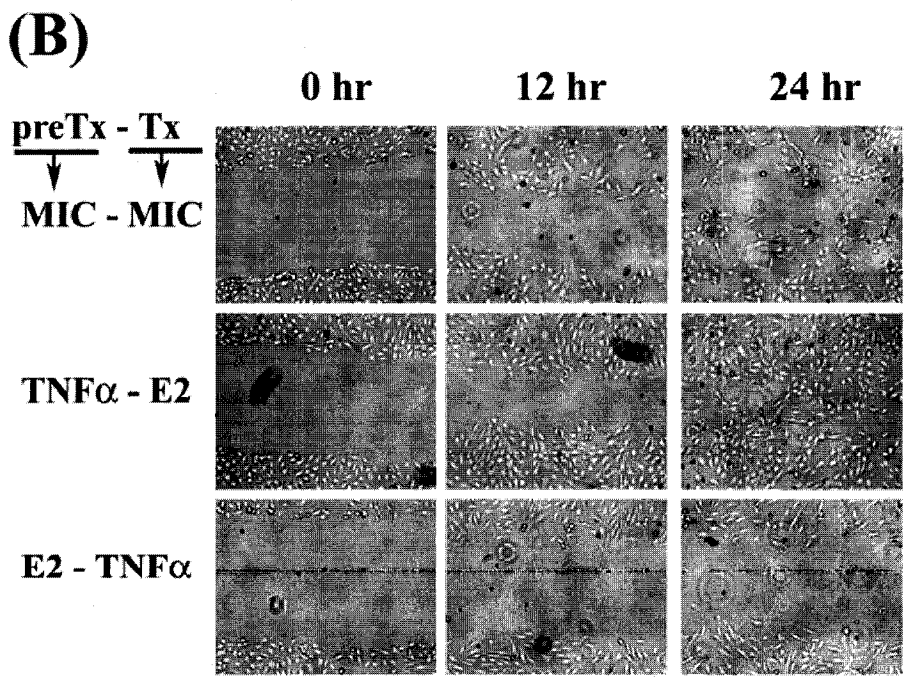


Figure IV-3. Estrogen increases migration in TNFα pretreated HUVEC.

(**A**). HUVEC were pretreated, wounded and subsequently treated in the following combinations (MIC-MIC, *diamond, dash-dot-dot*; MIC-E2, *hexagon, long-dash*; MIC-TNFα, *triangle down, dash-dot*; E2-TNFα, *triangle up, x-hair, solid line*; TNFα-E2, *circle, x-hair, solid line*) and quantified at 0, 6, 12 and 24 hours. (**B**). Example of a series of photomicrographs of HUVEC subjected to pretreatment (Pre-Tx) for 4 hours, wounding and treatment (Tx). A dynamic of the healing of representative HUVEC cultures is shown. (n = 3)

Proliferation of HUVEC treated with E2 and TNFα was dose-dependent

E2 induced a small but significant increase in DNA synthesis of HUVEC after 24 hours of treatment (*Fig. 4*). 0.3 and 1.0 ng/ml of TNFα suppressed the EC proliferation. The addition of E2 to TNFα 1.0 ng/ml prevented this decrease.

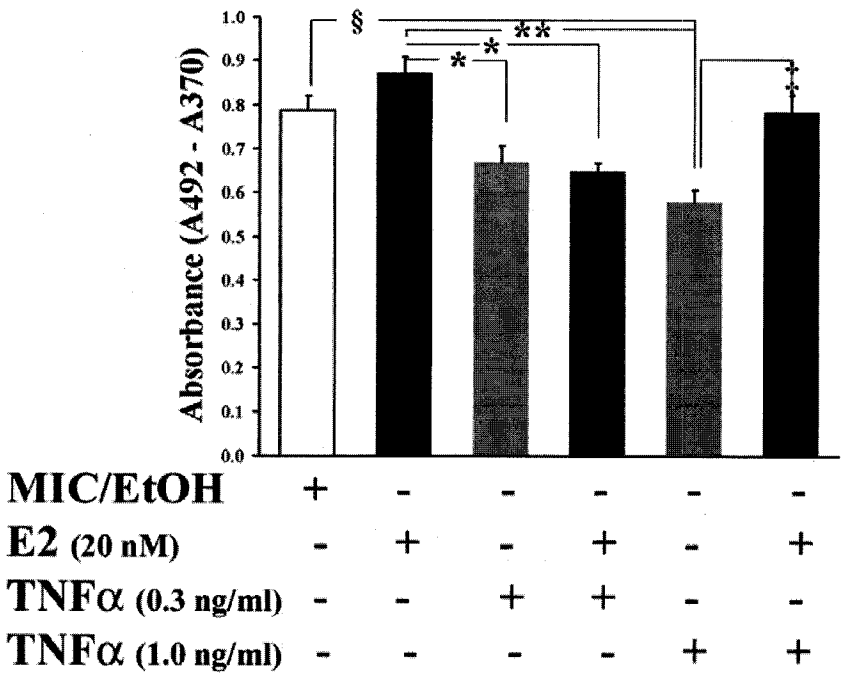


Figure IV-4. Assessment of proliferation in HUVEC with BrdU.

HUVEC labeled with BrdU were incubated for 24 hours in a conditioning media with solvent (MIC/EtOH), with Estradiol (E2) alone or in combination with 0.3 or 1.0 ng/ml of TNFα. E2 alone or combined with TNFα 1.0 ng/ml significantly increased the proliferation of HUVEC, in contrast to TNFα alone. Significance (One-way repeated measure ANOVA): *, § $P< 0.05$; **, ‡ $P< 0.001$. (n = 3).

E2 increased the direct cell count

The E2 alone, and when added to 0.3 or 1.0 ng/ml of TNFα, significantly increased the number of viable cells to 7.6 x 10^5/ml, 7.9 x 10^5/ml or 7.8 x 10^5/ml respectively (*Fig. 5*). Neither 0.3 nor 1.0 ng/ml of TNFα alone altered the number of viable cells compared to control (6.3 x 10^5/ml, 6.5 x 10^5/ml, 6.4 x 10^5/ml respectively).

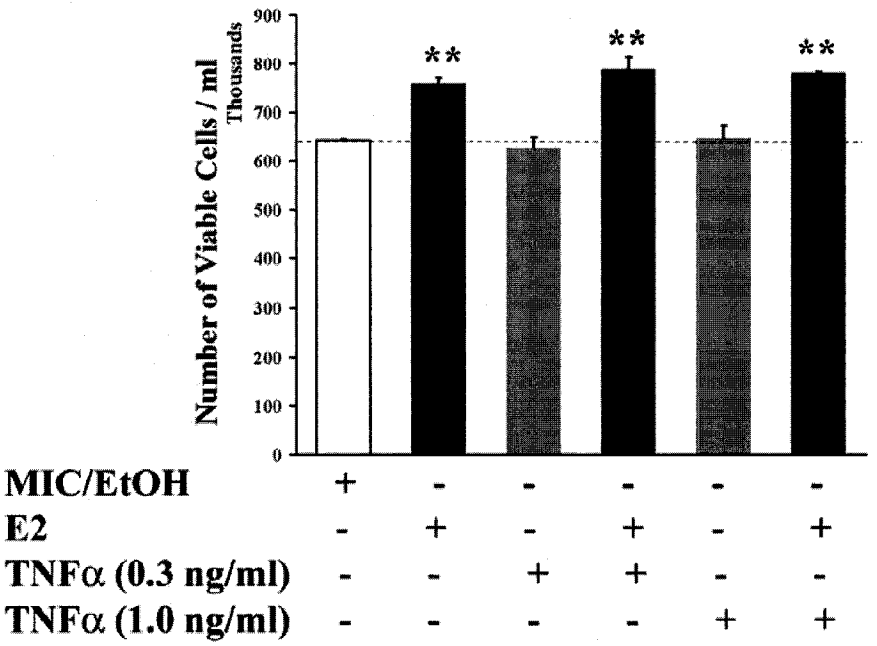


Figure IV-5. E2 increased the direct count of viable HUVEC.

HUVEC were incubated 24 hours in stimulating media containing only solvent (MIC/EtOH), with agonists E2, TNFα (0,3 or 1.0 ng/ml) or in combination. Viable cells were counted by Trypan blue exclusion. E2 alone or added to either concentration of TNFα, increased the number of cells. Significance by ANOVA, SNK test: ** P< 0.001.

Pro-angiogenic effects of E2 in HUVEC can be mediated by NO production

Because it was shown that NO plays an important role in promoting endothelial migration, survival and angiogenesis, and that E2 is known to increase NO in EC, we asked if the increased migration we detected in E2 treated HUVEC could be mediated by NO. Indeed, the E2 treatment (*Fig. 6 A*) increased the migration of wounded HUVEC by 33.3 ± 0.3 % and by 49.2 ± 2.8 % at 9 and 12 hours respectively. In the same time points, the untreated control (MIC) migrated only by 20.6 ± 1.3 % and 22.7 ± 1.0 %. The addition of L-NAME, the NOS inhibitor, abrogated the E2 effect so that the migration of HUVEC decreased significantly to 19.6 ± 0.8 % and 25.1 ± 2.2 % at 9 and 12 hours respectively. TNFα (*Fig. 6 B*) increased HUVEC migration by 24.7 ± 1.5 and by 29.6 ± 0.3 % at 9 and 12 hours respectively, while the co-treatment with E2 increased the migration to 28.1 ± 0.5 % and to 32.8 ± 2.3 %. The migration of EC with NO inhibitor L-NAME, however, decreased only by 23.2 ± 1.2 % and 28.6 ± 2.6 % in TNFα treated cells and by 27.4 ± 0.2 % and 28.8 ± 1.2 % in cells with combined treatment of TNFα with E2. Thus, TNFα prevented the E2 induced NO production.

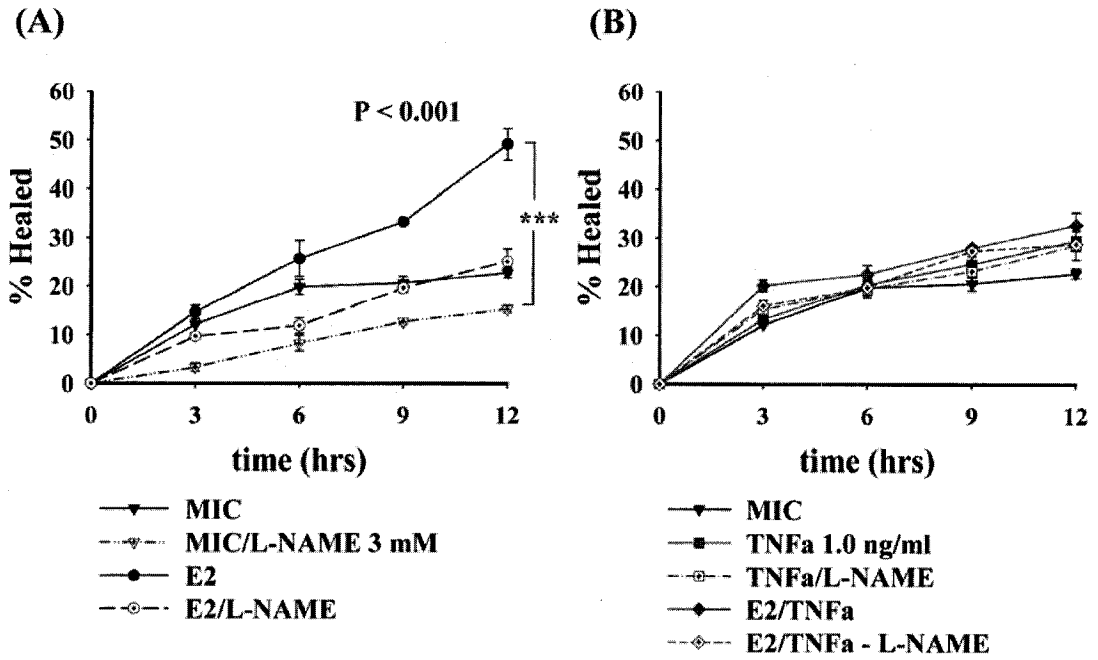


Figure IV-6. E2 increased the migration of HUVEC through generation of NO.

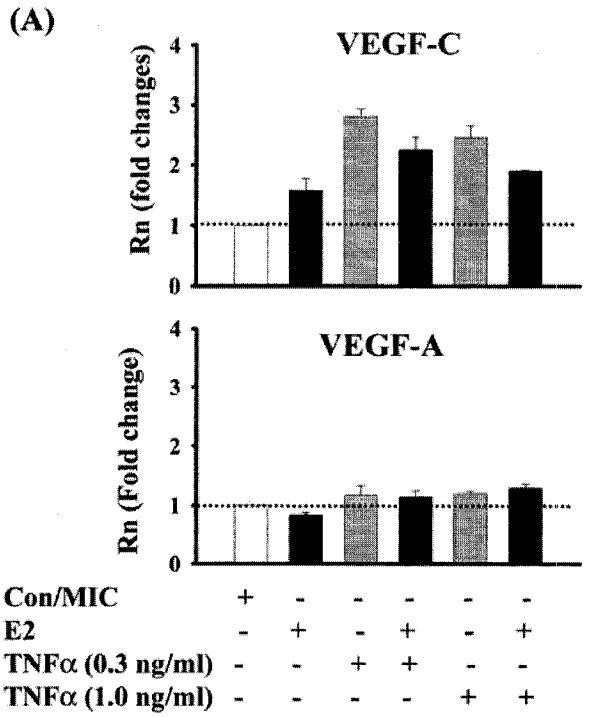
Wounded HUVEC were **(A)** treated in media alone (MIC) (*full triangle down*), media with L-NAME (*open* x-*hair-triangle down, dash-dot-dot line)*, with E2 (20 nM) alone (*full circle,solid line*), E2+L-NAME (3 mM) (*open* x-*hair-circle, dashed line*) or **(B)** with TNFα 1.0 ng/ml (*full square, solid line*), TNFα+L-NAME (*open x-hair-square,dash-dot line*), E2+TNFα (*full diamond, solid line*) or E2/TNFα+L-NAME (*open x-hair-diamond, dashed line*). The intensity of migration of HUVEC was quantified microscopically at indicated time points. N = 3; the significance by one-way RM ANOVA is *** $P<0.001$.

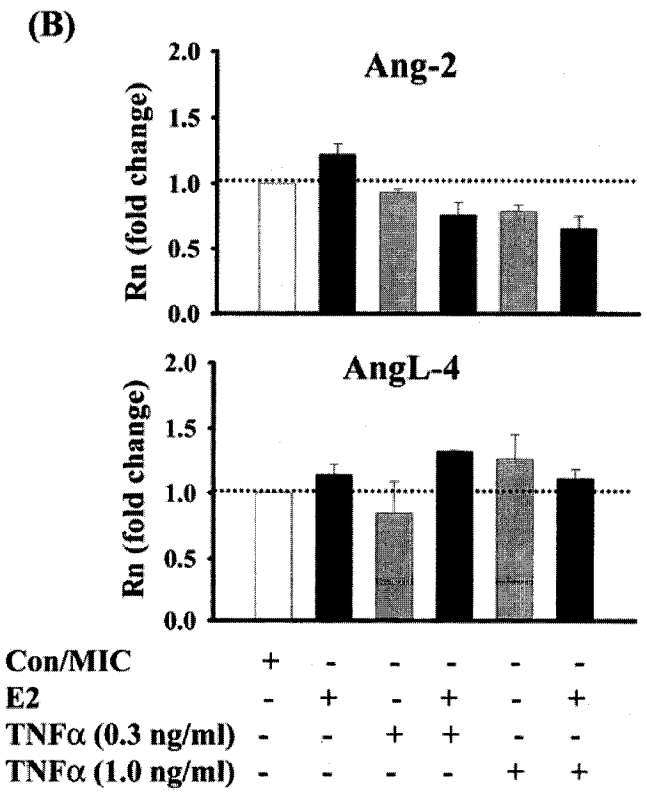
E2 and TNFα influenced the expression of angiogenesis-related genes

We performed real time PCR on HUVEC treated for 6 hours (*Fig. 7*). E2 increased VEGF-C, Ang-2 and Anglike-4 to 1.57 ± 0.22, 1.22 ± 0.8 and 1.14 ± 0.8 fold respectively above the control whereas VEGF-A decreased to 0.84 ± 0.4 fold of control (*Fig. 7 A,B*). In contrast to E2, TNFα tended to decrease Ang-2 and the addition of E2 did not reverse this. Both concentrations of TNFα (0.3 and 1.0 ng/ml) markedly increased VEGF-C to 2.81 ± 0.13 and 2.46 ± 0.19 fold of the control respectively. E2 partially prevented this effect to 2.25 ± 0.21 and to 1.9 ± 0.1 fold in TNFα 0.3 and 1.0 ng/ml respectively.

Both concentrations of TNFα 0.3 and 1.0 ng/ml significantly increased the IL-8 expression, to 32.83 ± 1.22 and 42.66 ± 3.58 respectively (*Fig. 7 C*). The addition of E2 decreased the TNFα-induced increase in IL-8 to 24.23 ± 3.44 and 33.54 ± 2.23 fold of the control respectively. VEGF-A was mildly increased above the control with E2 but also with TNFα.

In summary, these results indicate that E2 blunts some of the signaling changes induced by TNFα but increases the promotion of migration and proliferation, so that the net effect is an increase of angiogenic response to TNFα.


(A)
VEGF-C
Rn (fold changes)
4
3
2
1
0
VEGF-A
Rn (Fold change)
4
3
2
1
0
Con/MIC + - - - - -
E2 - + - + - +
TNFα (0.3 ng/ml) - - + + - -
TNFα (1.0 ng/ml) - - - - + +


(B)
Ang-2
Rn (fold change)
2.0
1.5
1.0
0.5
0.0
AngL-4
Rn (fold change)
2.0
1.5
1.0
0.5
0.0
Con/MIC + - - - - -
E2 - + - + - +
TNFα (0.3 ng/ml) - - + + - -
TNFα (1.0 ng/ml) - - - - + +

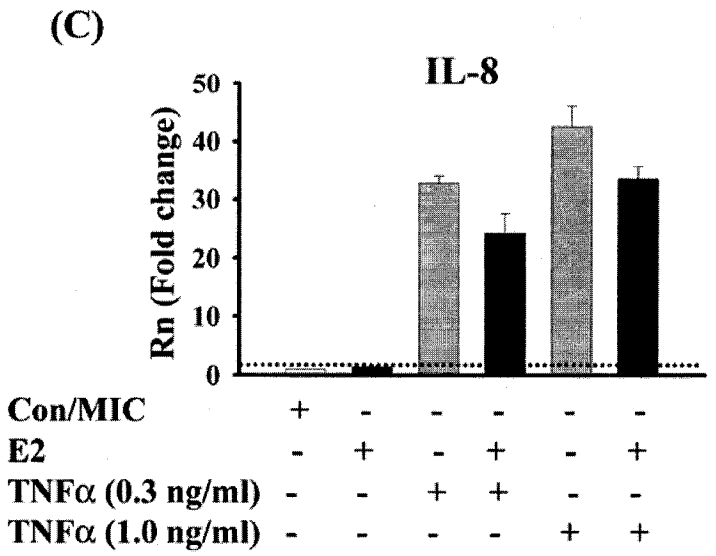


Figure IV-7. E2 influence on inflammation and angiogenesis related genes.

HUVEC were wounded at the beginning of stimulation and incubated for 6 hours in growth-factors and steroid depleted serum (Control, MIC), or with E2 20 nM, TNFα 0.3 or 1.0 ng/ml or in combinations. The induction of gene relative quantification was determined by real-time PCR for the expression of VEGF-C, VEGF-A (**A**) Ang-2, AngL-4 (**B**) and IL-8 (**C**). (n = 2).

Activation of PAK1

The p21-activated kinase-1 (PAK1) is a downstream effector protein of PI(3)K that was found to be induced by growth factors in breast cancer cells and VSMCs (595-597). E2 was also shown to activate the PAK1 pathway (598). Although PAKs (a family of serine/threonine kinases) are effectors of the small GTP-ases, it was found that PAK1 mediates cell migration via its effect on the cytoskeleton, which has been reported to be largely independent of the Rho GTP-ases (599).

We studied the possible activation of the PAK1 signaling pathway by E2 and TNFα 1.0 ng/ml. We found increased PAK1 phosphorylation at $Ser^{199/204}$ after 1-hour stimulation with TNFα alone, or combined with E2 or in HUVEC pretreated with TNFα and subsequently treated with E2 (*Fig. 8, top rows*).

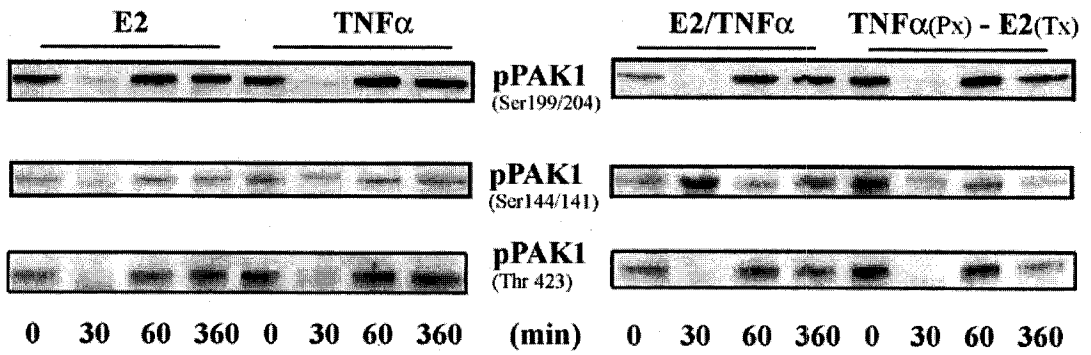


Figure IV-8. Pretreatment (Px) with TNFα followed by treatment (Tx) with E2 significantly phosphorylated the PAK1 on Ser199/204 at 60 minutes.

HUVEC were treated with with E2 20nM, TNFα 1.0 ng/ml alone, in combination of both or pretreated with TNFα for 4 hours and subsequently treated with E2 at 0, 30, 60 or 360 min. Phosphorylation of PAK1 at serine 199/204, 144/141 or threonine 423 residues was examined. The probing for total PAK1 has currently been undertaken.

IV.5. DISCUSSION

The major findings of this study were that E2 increased endothelial cell migration, an early step in angiogenesis. TNFα in the concentration of 1.0 ng/ml also demonstrated a proangiogenic effect in HUVEC and the effect of TNFα was potentiated by E2. We also found a modification of the expression of angiogenesis-related genes, VEGF-C by E2 and IL-8 by TNFα.

The increase of migration in wounded HUVEC by E2 was previously shown by Morales (192). Cells cultured in E2 achieved greater wound closure within 24 hours than did untreated cells.

The finding that TNFα also positively influenced the EC wound repair and that this was potentiated by mitogens has also previously been shown. TNFα increased migration and wound healing of HUVEC elicited by VEGF-A in time and dose-dependent manner demonstrating its angiogenic effects in ECs (431). In our study it was the mitogen E2 alone that initiated the most intensive wound repair in scratched HUVEC. The angiogenic effects initiated in wounded HUVEC by TNFα were potentiated by E2.

We demonstrated by a wound injury/migration assay that E2 increases angiogenesis induced by TNFα.

Mechanical denudation in nearly confluent endothelial cell cultures elicited a regenerative reaction, which resulted in the repopulation of the monolayer. The wound healing process appeared to involve both cellular migration and proliferation. In wound-injured cultures, the initial response was cellular spreading and migration from the wound edges into the denuded area. Migration was underway by 12 hours, however there were significant differences between treatments. In particular, the high dose (20 ng/ml) of

TNFα demonstrated only sparse migration of cells into the scratched area even after 24 hours whereas E2 alone (*Fig. 1 A,B*) already strongly reconstituted the wounded area after 12 hours. The medium dose (1.0 ng/ml) also partially increased migration whereas the low dose (0.3 ng/ml) of TNFα did not. The addition of E2 potentiated this effect of medium dose of TNFα. Furthermore, pretreatment with TNFα followed by E2 treatment dramatically increased EC migration. The significance of these observations is important. When E2 is given in the presence of active inflammatory molecules that stimulate chronic inflammation such as TNFα, the proangiogenic effects of E2 can amplify the proangiogenic and prothrombotic effects of TNFα.

Hormone replacement treatment in E2 deficiency has been shown to decrease the expression and secretion of the proinflammatory cytokine IL-1 (600) and TNFα (601) in cultured monocytes from blood of postmenopausal women. Circulating TNFα also was found in increased levels after natural or surgical menopause (602). However, an equally large number of studies failed to demonstrate any effects of E2 on circulating levels of these cytokines (603;604). Some studies showed biphasic (605) or conflicting data (606) for the effects of E2 on the concentration of circulating TNFα. Nevertheless, the relation between E2 and cytokines, notably TNFα, is more complex (607).

Amplification of the inflammatory response of TNFα could occur through two general processes. First, TNFα will likely decrease eNOS (608) and increase ROS (609), which are two beneficial effects of E2 (610). Chen and coworkers recently found that the addition of Ang-2, which acted through Tie-2, increased TNFα-induced angiogenesis (611). We showed that E2 increased Ang-2 and abrogated Tie-2 autophosphorylation in OVX rats and decreased Ang-1 and thus produced a pro-angiogenic phenotype (470).

Therefore, E2 could induce Ang-2 and work synergistically with TNFα to promote angiogenesis.

When we evaluated mRNA expression of IL-8, a mediator of inflammatory reactions and angiogenesis (612), we found that E2 did not enhance gene expression. TNFα however, strongly induced it and E2 partially suppressed this induction. Our data are consistent with others. TNFα promoted the proliferation of endometriotic stromal cells by inducing IL-8 (613). Endometriosis is considered to be an inflammatory disease. Addition of E2 did not have much effect on IL-8 mRNA expression perhaps because of lower levels of ERs in these cells, which were also exposed to treatment with gonadotropin-releasing hormone (614). In contrast to our findings, others reported no change in IL-8 mRNA expression in HUVEC treated with TNFα, E2 or with a combination of these. However, secretion of IL-8 into the culture supernatant was enhanced by TNFα and this was partially inhibited by treatment with E2. Thus, E2 blocked the secretion but not the gene transcription of this proinflammatory chemokine (483). We found regulation of IL-8 mRNA by both TNFα and E2; this could be due to the fact that we monitored the dynamics of production of IL-8 mRNA in real time, while they evaluated only the final amount of IL-8 cytokine.

VEGF plays a pivotal role in the regulation of normal and pathological angiogenesis. VEGFs are secreted proteins, in contrast to other EC mitogens such as FGF and platelet derived endothelial cell growth factor (422). VEGF is a potent mitogen for vascular endothelial cells. VEGF acts through two tyrosine-kinase receptors, Fms-like tyrosine kinase-1 or VEGF receptor-1 (flt-1)(VEGFR-1) and fetal liver kinase/kinase-insert domain receptor or VEGF receptor-2 (flk-1/KDR)(VEGFR-2), which are expressed

almost exclusively in EC (615). Several studies have shown that VEGFR-1 (616) and VEGFR-2 mRNAs (617;618) are expressed constitutively by ECs in culture (618;619). VEGF-C was identified as a ligand for VEGFR-2 and the new flt-4 receptor (VEGFR-3). In addition, VEGF-C stimulated the migration of bovine capillary ECs (425).

We show that VEGF-C is mildly upregulated by E2 after 6-hour treatment. In a study on VEGF-C expression in MCF7 human breast cancer cells treated with E2, abundant VEGF-C mRNA expression at the baseline dropped within the first 3 hours after E2 treatment but then increased by 4 hours (620). This is consistent with our finding of moderately increased VEGF-C gene expression after 6-hour treatment with E2 in HUVEC. In one study E2 upregulated the VEGF-C receptor VEGFR-2 (KDR) in microvascular endothelial cells (MEC) isolated from myometrial tissue. The increase in VEGFR-2 (KDR) expression was observed only in ERα-expressing MEC isolates and not in ERα-negative samples. As well, the antiestrogen ICI 182,780 inhibited VEGF-binding and MEC-proliferation in E2-treated ECs. Therefore the increase in VEGFR-2 expression was mediated by E2 through ERα (469).

We found that VEGF-C was also induced by TNFα. The increase of VEGF-C by TNFα was almost a 3-fold. *In vivo*, the angiogenic activity of TNFα has been demonstrated in the rabbit (621) and rat cornea (622). *In vitro*, TNFα at very low concentrations (3.5 ng/ml) stimulated capillary tube formation in bovine adrenal capillary ECs (622). These data from literature showed a rather unexpected finding that TNFα is an agonist of neovascularization (621;623). TNFα also increases VEGFR-2 mRNA in HUVEC (431). In this regard, the fact that in our experiments TNFα enhanced VEGF-C could be explained by the induction of VEGFR-2, since the latter is known to create a positive

loop with VEGF-C, perhaps by autophosphorylation through the MAP kinase cascade (624), and subsequent proangiogenic effects in ECs. The VEGFR-2 regulation by TNFα, however, is not consistent throughout the literature. For instance, another group of researchers demonstrated that TNFα downregulated VEGFR-2 expression in HUVEC (625).

We found that VEGF-A mRNA was at a level of the baseline in 6-hour treated HUVEC by E2. Our result of an unchanged VEGF-A level after E2 treatment could be explained by the fact that the angiogenic effects of VEGF-A are mediated mostly by VEGFR-1 which has a decoy effect on VEGF signaling in the vascular endothelium and prevents its interaction with VEGFR-2 (626;627). In endothelium, VEGFR-1 releases tissue-specific growth factors (628). The data from our experiments led us to speculate about the known fact that the main receptor involved in EC proliferation, migration and survival is VEGFR-2 (629), which mediates the release of its ligand VEGF-C through the positive feedback (425).

Using real-time PCR, we also examined the effects of E2 and TNFα on AngL-4 gene expression in HUVEC. We found only nonsignificant modifications of this angiogenic factor and these changes were above the baseline by E2 treatment alone. Altough Ang-4 is known to play a role in angiogenesis (630), neither aforementioned findings nor our data so far support the evidence for direct regulation of Ang-4 by E2 in HUVEC.

We found increased phosphorylation of PAK1 after 1-hr treatment with TNFα and E2 at $Ser^{199/204}$ residues. Since PAK1 is an important modulator of cell migration and proliferation, this finding seems to be consistent with the results of the migration assay, particularly in TNFα-pretreated and E2 treated HUVEC. It could signify increased

proangiogenic effects. These results must be interpreted carefully; for instance, an overall deletion of pPAK1 after 30 minutes of treatment may be caused by stress associated with the changing of media.

The role of NO in angiogenesis has been questioned. Chemically derived NO inhibited serum-induced migration of HUVEC (476). L-NMMA, an eNOS inhibitor, increased vascular density in the chick embryo chorioallantoic membrane (477). *In vitro*, exogenous NO but also a cell permeable cGMP analogue reduced tube formation in the matrigel tube formation assay (478). On the other hand, more recent studies showed the importance of NO in the angiogenic response to VEGF. Indeed, NO mediated the VEGF-induced proliferation in HUVEC and stimulated network structures in an NO-dependent manner. VEGF induced increase in the NO production was mediated by activation of tyrosine and PI(3)-kinases (479). In support of these data, in another study endogenous endothelium-derived NO maintained the functional expression of integrin, a mediator for endothelial migration, survival and angiogenesis (480). In a mouse model with targeted disruption of eNOS gene, investigators showed that vascular injury caused an increase in neointimal proliferation. This finding was consistent with a protective role of eNOS in the prevention of vascular intimal formation *in vivo*. Furthermore, pregnancy suppressed the response to vessel injury in both wild-type and eNOS mutant mice suggesting that this effect is due to circulating estrogens (481).

E2 is known to increase NO in EC. Our data greatly confirmed that the increased migration of E2 treated HUVEC was due to the presence of increased production of NO since the specific eNOS inhibitor L-NAME suppressed this effects of E2 on migration of EC. The increase of migration of EC with TNFα or combined with E2 was much lower

compared to that with E2 alone and was very little reduced by the eNOS inhibitor. Thus, TNFα prevents the E2 induced of NO production and thus negates the antiinflammatory benefits of E2. The increase in angiogenesis from combined actions of E2 and TNFα could be harmful when atherosclerotic plaques are advanced. The neovascularization will create the plaque instability and lead to its rupture. In such case, the prothrombotic effects of E2 can promote thrombosis on the denuded surface of vessels. Also, because of lack of endothelium in these denuded areas, E2 does not stimulate the NO production nor activate AKT. These are contribution factors that can lead to deteriorated situation in the vascular wall.

In summary these findings indicate that E2 blunts some of the signaling changes induced by TNFα but that it increases the promotion of migration and proliferation so that the net effect is to increase the angiogenic response to TNFα.

V. General Discussion

Limitations of EC culturing

Cell culture disturbs ECs from their quiescent in vivo state characterized by 0.1% replications per day to an activated phenotype with 1% to 10% replications per day accompanied by loss of specialized functions associated with diverse vessels and organ systems (34).

Human EC loose their phenotype after only 2 days in culture as shown by DNA microarray analysis of postcapillary endothelial venule ECs and HUVEC (631).

Differences in the culture systems used for experiments were reported by a group of investigators that were unable to reproduce the induction of IL-8 in HUVEC isolated in their own laboratory (632) whereas the similar experiment on HUVEC from commercial source responded to human IL-8 as a mitogen (612). Thus, isolation procedures and culture conditions have to be considered for different studies.

The choice of cell type

Studies were done primarily in HUVEC. They are the "industry standard" (138;447;448;519;633) and they are widely used in basic vascular research. HUVEC thus provide a good model to study the biology of estrogens in anticipation of clinical application. They have numerous advantages. HUVEC are a primary mature human cell line. They are available and grow at reasonable pace. However, they can also give variable responses because of differences in the phenotypes of the donors and changes in phenotypes with passages. There is a long experience with them in our laboratory. These

cells are also from a conductance vessel, which is the type of vessel in which atherosclerosis occurs. They have strong expression of ERβ mRNA and weaker and more variable ERα (*Fig. 1*) and express both ERα and ERβ protein (*Fig. 2*). Coworkers have also fully characterized their gene expression in response to TNFα (518) and have experience with their response to Ang-1 and Ang-2 (447;448;633). I have also had previous experience with this cell type (138;519).

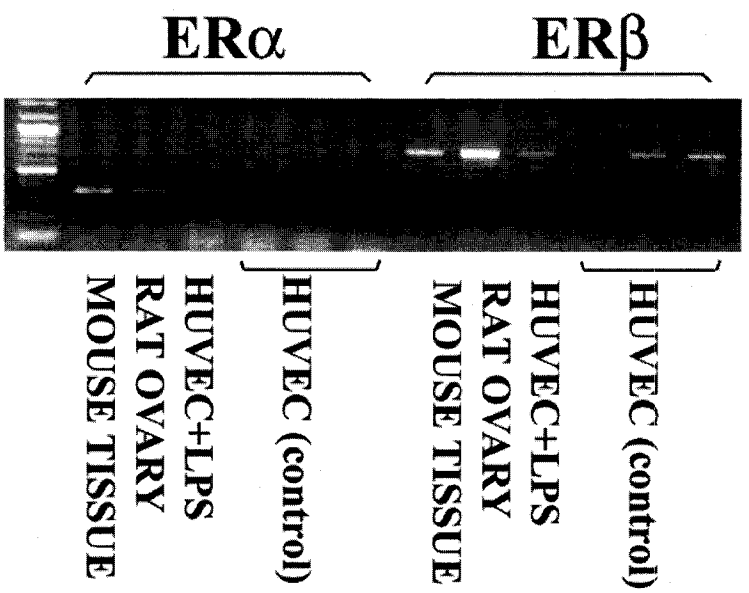


Figure V-1. ERα and ERβ mRNA expression.

Determination of ERα (356 bps) (left side) and ERβ (777 bps) (right side) by PCR in untreated HUVEC (control) and HUVEC stimulated with LPS. Rat ovary and mouse tissue served as positive controls.

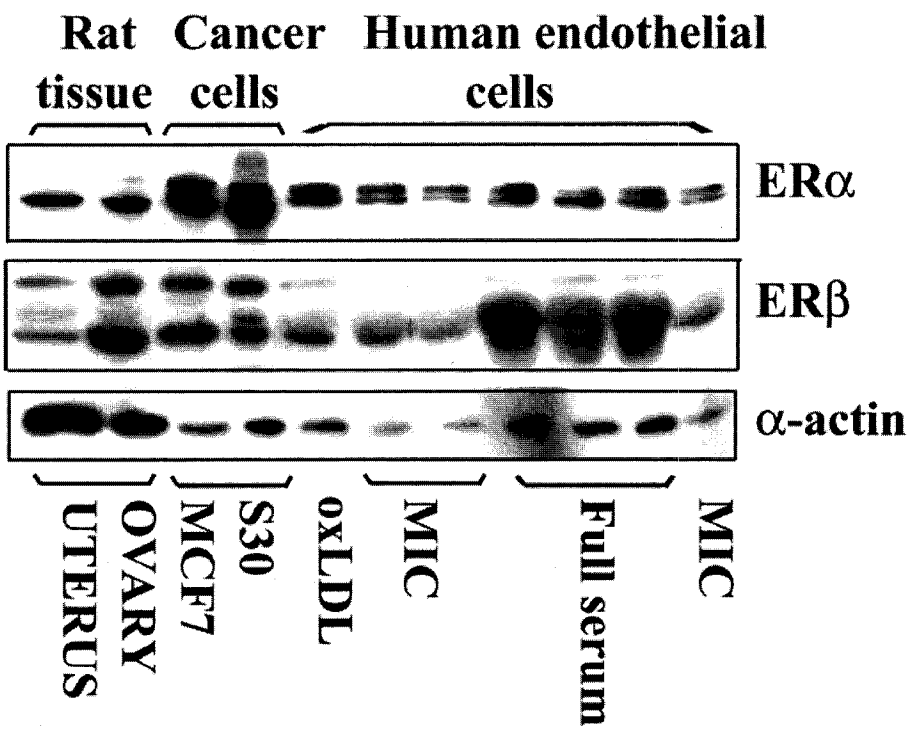


Figure V-2. Estrogen receptors protein.

ERα (top row) and ERβ (middle row) were detected by immunoblotting in HUVEC cultured under different conditions: in medium with charcoal stripped serum (MIC), in medium with 15% FBS (full serum) or were conditioned with oxLDL, 75 μg/ml. Human breast cancer cells S30, stably transfected with ER, estradiol dependent MCF7 cells and rat gonadal tissue ovary and uterus served as positive controls.

General Discussion

Cardiovascular disease (CVD) is a leading cause of mortality in postmenopausal women. The loss of estrogen (E2) that accompanies menopause contributes to the development of CVD and E2 is known to protect women against heart disease. Conflicting results exist in the literature regarding actions of E2 on the cardiovascular health.

Recently, NAD(P)H oxidase has attracted considerable attention as a contributor to the pathogenesis of several cardiovascular disease including atherosclerosis and hypertension (292;324). Studies in mice that are deficient in components of NAD(P)H oxidase notably $p47^{phox}$ and $gp91^{phox}$ (also known as NOX2) show that reactive oxygen species (ROS) produced by these oxidases contribute to endothelial dysfunction. Our study reveals that estrogen influences the expression and function of NAD(P)H oxidase within the rat cardiovascular tissue. E2 replacement prevents an ovariectomy-induced increase in vascular superoxide (O_2^-) release. We also found that NAD(P)H oxidase inhibitor diphenyleneiodonium (DPI) abolished the O_2^- production, which supports a role for NAD(P)H oxidase in O_2^- production. However, we found that E2 did not alter NADH or NAD(P)H stimulated O_2^- production, which suggests that the amount of NAD(P)H oxidase does not change. Our results are in contrast with recently published data of Miller et al who found lower O_2^- production in NAD(P)H-stimulated basilar cerebral arteries of females compared to males (634). Thus E2 appears to have a more regulatory role for NAD(P)H oxidase. This regulatory role of E2 in our study is reinforced by the fact that E2 decreases in O_2^- production are associated with a decrease in $p47^{phox}$ membrane component of the NAD(P)H oxidase.

In contrast to the studies of Arenas et al. who found modulation of vascular function in E2-depleted rats (341), we did not find that the reduction of O_2^- with E2 treatment or acute radical scavengers or inhibitors of O_2^- production by NAD(P)H had any effect on vascular function. This was true in our study where the decrease in O_2^- production was not associated with a change in the contractile response to phenylephrine and dilation in response to acetylcholine (Ach) or superoxide dismutase (SOD). Finally, endothelial nitric oxide synthase (eNOS) expression was not increased by the treatment with E2. There was no evidence for a change in the bioavailability of nitric oxide (NO). Thus, in agreement with previous (333;635) and recent studies (634), E2 action on endogeneous NO does not appear to regulate the concentration of O_2^-.

Our study advances the understanding of both the role of O_2^- in mediating endothelial damage leading to atherosclerosis and the mechanisms by which E2 provides vascular protective effects. We can agree on estrogen's ability to reduce the generation of O_2^- that has been demonstrated in vitro and in numerous animal models. E2 indeed diminishes the NAD(P)H oxidase protein expression. However the expectation that chronic E2 replacement could be also an efficient modulator of vascular tone by increasing the bioavailable NO does not appear to be valid. Rather, E2 maintains endothelial health by reducing the oxidative stress and improving endothelial NO/O_2^- balance, and therefore controlling local concentrations of NO. Although we found no change in bioavailability, a study by Wassmann (636) showed that the effects of E2 on the inhibition of NAD(P)H oxidase activity prolong the NO half-life and may have thus protective effects against the development of atherosclerosis.

A mechanism for the ability of E2 to decrease the oxidative stress in the vasculature is given by our finding of the decrease of $p47^{phox}$ in endothelial cells. Thus, E2 reduced the expression and localization of a component of the non-phagocytic NAD(P)H oxidase known to maintain constitutive and permanent O_2^- production. E2 acts as an indirect antioxidant at the genomic level by reducing the capacity of endothelial cells to generate O_2^-.

It is possible that the estrogen-induced decrease in O_2^- production could alter intracellular signaling and thereby contribute to the vascular protection observed with estrogen treatment. This could improve vascular health particularly in postmenopausal women deprived of effects of E2. It is likely that when atherosclerotic lesions are more advanced, these positive effects of E2 are too weak to alter the cause of the disease and thus would only be helpful in maintaining normal endothelial health or reversing early stages of atherosclerosis.

Apoptosis of cells in atherosclerotic lesions is thought to contribute to plaque destabilization and cardiovascular events (494;544;550). Since estrogens have anti-apoptotic properties in endothelial cells (394), cancer cells (128;404), as well as in vivo (496), E2 could be vasculoprotective. We thus studied the potential for E2 to reduce apoptosis in endothelial cells treated with tumor necrosis factor alpha (TNFα) and oxidized low density lipoprotein (oxLDL), two known proapoptotic substances that play a role in the progression of atherosclerosis (551). Spyridopoulos et al previously showed that E2 treatment reduces TNFα induced apoptosis in endothelial cells (394). E2 has also been shown to reduce endothelial apoptosis induced by serum deprivation (504).

We studied oxLDL because of its pathological importance. OxLDL plays an important role in atherosclerosis (506;562) and indeed oxLDL has been shown to induce endothelial apoptosis (496;500;510;552;553;555;563-566). The effect of E2 on oxLDL- induced apoptosis has not been previously studied and could potentially be an important part of the vasculoprotective effects of E2. We found that E2 did indeed reduce oxLDL induced apoptosis and the magnitude of that reduction was very similar to the reduction of TNFα induced apoptosis by E2.

Some researchers (554) found that the antiapoptotic effect of TNFα and oxLDL is not due to altered expression of Bcl-2 and Bcl-xL, although one group did find that oxLDL decreased bcl-2 expression in endothelial cells (564). On the other hand, we show that E2 treatment increased Bcl-2 and Bcl-xL expression, which could have contributed to its antiapoptotic effects. In support of this finding, Pike et al found that E2 increased Bcl-xL expression in cultured hippocampal neurons and identified an estrogen response element in the bcl-x gene (572).

E2 treatment reduced the action of the final executive member of the apoptotic pathway, cleaved caspase-3. After 24 hours, E2 induced Bcl-proteins, notably Bcl-xL. TNFα seemed to act by extramitochondrial pathways, without direct interactions with the Bcl-2 and Bcl-xL proteins, and oxLDL even induced Bcl-proteins, particularly Bcl-xL at 24 hours. This accounted for the fact that the stimulating effects of E2 on Bcl-proteins were partially masked by oxLDL. We also showed that in the short time delay of 1 hour the E2 induced the phosphorylation of BAD, the proapoptotic Bcl-protein. Furthermore, Wortmannin, the PI(3)K inhibitor, blocked the beneficial effect of E2 on endothelial cells

by increased NO through PI(3)K/Akt cascade mediated by phosphorylation of eNOS (138).

We focused our study on the mechanisms of E2 protection under conditions that occur in a stage of developing a vascular damage. However, it is well known that E2 may act through numerous mechanisms, genomic and non-genomic, to exert protection of the endothelium. Our data show that E2 prevents the delayed pattern of cell death. E2 not only decreased apoptosis of endothelial cells triggered by both oxLDL and TNFα only after long time exposure, but also the induction of prosurvival antiapoptotic proteins Bcl-2 and Bcl-xL occured only after hours of treatment. This means that estrogen replacement treatment takes time to take effects. In contrast, prosurvival effects of E2 on BAD phosphorylation was rather immediate, suggesting E2 non-genomic actions perhaps through PI(3)K/AKT pathway and possible acute changes in NO.

Although E2 reduces TNFα and oxLDL induced apoptosis in HUVEC to a similar degree, the reduction of apoptosis produced by both agonists was not large so that the net in vivo effect would depend on the balance between the prevention of apoptosis and the strength of the pro-apoptotic signal. Furthermore, E2 did not alter early apoptosis induced by TNFα and only reduced the apoptosis seen at 24 hours. Our results suggest that the action of E2 requires the induction of anti-apoptotic proteins or only acts upon factors that act later in the apoptotic process. As we found with the reduction of superoxide by estrogen, these results would mean that the endothelium would have to be functional and able to produce anti-apoptotic factors, which might not be the case in advanced atherosclerotic lesions.

E2 is known to inhibit atherogenesis by inhibiting many of the early steps of atherosclerotic plaque formation. However, the lack of vasculoprotective effect by postmenopausal hormone replacement therapy (HRT) and possible increase in cardiovascular disease (6;7;14) observed during the first year after the initiation of HRT suggests that once the plaque is formed, E2 may have additional effects that may counteract its beneficial outcomes. Neovascularization as a consequence of the angiogenic properties of E2 explains the adverse effect of E2 on plaque stability *in vivo* (29).

We found that in wound injury/healing assay E2 increased migration of HUVEC time dependently. A low dose of TNFα increased the migration of EC as well, and this increase was potentiated by E2. However the overall increase of E2 combined with TNFα did not achieve the intensity of migration with E2 alone. TNFα decreased the proliferation of HUVEC at 24 hours but E2 rescued this process. Furthermore, pretreatment with TNFα followed by E2 treatment dramatically increased EC migration. The significance of these observations is important. When E2 is given in the presence of active inflammatory molecules that stimulate chronic inflammation such as TNFα, the proangiogenic effects of E2 can amplify the proangiogenic and prothrombotic effects of TNFα.

The question we addressed here is relevant to health in the population of postmenopausal women. It may explain at least partially why large trials have failed to confirm benefits of estrogen replacement therapy (ERT) on lowering a risk of CVD and even showed the contrary effects. Indeed, this may be due to the proangiogenic capacity of E2 which

effects already damaged or inflamed endothelial plaques. Furthermore, proangiogenic effects of E2 may contribute to the higher incidence of breast cancer in women on ERT.

The mechanism of estrogen's promoting effect on migration of endothelial cells, an early stage in angiogenesis (192), is not yet known. Nitric oxide (NO) could be one of the factors that mediate the increase in angiogenesis by E2 (479-481). Indeed, in our data, the implication of eNOS that generates NO is evident, since L-NAME, the inhibitor of eNOS antagonized the promigratory effects of E2. In contrast, we did not observe similar effects on TNFα treated cells, which suggests that proangiogenic and proinflammatory effects of TNFα require another mechanism than increases in NO.

On the other hand, proinflammatory cytokines such as TNFα generate reactive oxygen species (ROS), notably superoxide in the vasculature. The O_2^-/NO balance determines the anti/proinflammatory phenotype assumed by the vasculature. Under normal conditions, the flux of NO generated by the endothelium greatly exceeds the production of O_2^-. The risk factor for CVD can result in the activation of cytokines that produce large quantities of superoxide (593;637) and lead to O_2^-/NO imbalance and induce an increase in adhesion of leukocytes and platelets as well as an increase in synthesis and surface expression of endothelial adhesion molecules. Since E2 promotes NO production it could limit the progression to a proinflammatory phenotype. But once again, this is likely only true at an early stage of atherosclerosis.

To explain the interacting angiogenic effects of E2 and TNFα we used HUVEC for convenience. In the future, a mouse model should be considered. Mice chronically expressing TNFα in the endothelium could provide a model of chronic inflammation. E2 status could be controlled by ovariectomy and ERT. This type of study would better

characterize the interaction of TNFα with E2 and at least indirectly approach the specific issue of plaque stability. An *in vivo* model of angiogenesis would help verify that *in vitro* responses correlate and give further insight into the more complex responses in the vasculature. Also, the knowledge of the intracellular signaling between the Tie-2/Ang-1 and Ang-2 with TNFα and E2 (470) could be further explored to enhance the understanding of the mechanism of action of these pathways in endothelium.

The clinical implication of these data is very important. It could explain, at least partially, why ERT has been unsuccessful in primary and secondary prevention trials. From our results is evident that the administration of E2 to women who have advanced atherosclerotic plaques and a high concentration of proinflammatory factors such as TNFα, would be unlikely to benefit and would certainly have worsening of their disease and increased cardiovascular events by inducing plaque instability. This would occur by E2 increasing neovascularization around atherosclerotic plaques. Furthermore, smooth muscle cells prevail on denuded areas of the vascular wall with damaged endothelium and E2 will increase their proliferation, which would also contribute to plaque formation. In healthy endothelium however, E2, perhaps by NO production, contributes to the maintenance of healthy endothelium. This is the paradox of the action of estrogen and a basis for the conflicting clinical results.

VI. Final Conclusions and Summary

In the present study we showed that chronic E2 treatment of ovariectomized rats decreased the production of O_2^- by the vascular wall. This was not associated with evidence of increased bio-available NO nor a change in vascular contractile properties. The decrease in O_2^- production was associated with a decrease in the membrane fraction of $p47^{phox}$, which suggests that E2 treatment alters O_2^- production by regulating the association of $p47^{phox}$ with the full NAD(P)H oxidase complex.

Our study advances the understanding of both the role of O_2^- in mediating endothelial damage leading to atherosclerosis and the mechanisms by which E2 provides vascular protective effects. We can agree on estrogen's ability to reduce the generation of O_2^- that has been demonstrated in vitro and in numerous animal models. E2 indeed diminishes the NAD(P)H oxidase protein expression. However the expectation that chronic E2 replacement could be also an efficient modulator of vascular tone by increasing the bioavailable NO does not appear to be valid. Rather, E2 maintains endothelial health by reducing the oxidative stress and improving endothelial NO/O_2^- balance, and therefore controlling local concentrations of NO. Perhaps an estrogen-induced decrease in O_2^- production could alter intracellular signaling and thereby contribute to the vascular protection observed with E2 treatment. This could improve vascular health particularly in postmenopausal women deprived of effects of E2. It is likely that when atherosclerotic lesions are more advanced, these positive effects of E2 are too weak to alter the cause of the disease and thus would only be helpful in maintaining normal endothelial health or reversing early stages of atherosclerosis.

We also showed that E2 inhibited apoptosis induced by TNFα and oxLDL in HUVEC. However, the inhibition was not complete. Whereas E2 decreased apoptosis induced by both, TNFα and oxLDL, there was an important difference in the timing of apoptosis induced by these two agents, and for both TNFα and oxLDL the action of E2 occurred at a later time point. E2 did not inhibit the early induction of apoptosis produced by TNFα. The delayed action of E2 on inhibition of apoptosis suggests the need for induction of factors such as the increase in Bcl proteins, Bcl-2 and Bcl-xL, which we observed, and possible effects on inhibitors of the death domain pathway such as FLIP. This inhibition of apoptosis in endothelial cells would potentially be vasculoprotective, however the inhibition of E2 in other cell types such as macrophages could promote atherosclerosis. Furthermore, the antiapoptotic effect of E2 on ECs can only occur if the cells can produce anti-apoptotic molecules in response to E2.

We found that E2 partially suppressed the proinflammatory effects of TNFα. On the other hand E2 increased the migration and proliferation and this effect potentiated an angiogenic response to TNFα. The mechanism of estrogen's promoting effect on migration of endothelial cells, an early stage in angiogenesis, is not yet known. Nitric oxide could be one of the factors that mediate the increase in angiogenesis by E2. Since E2 promotes NO production it could limit the progression to a proinflammatory phenotype. But once again, this is likely only true at an early stage of atherosclerosis. From our results is evident that the administration of E2 to women who have advanced atherosclerotic plaques and a high concentration of proinflammatory factors such as TNFα, would be unlikely to benefit and would certainly have an aggravating effect on their disease. Furthermore, it would increase cardiovascular events by inducing plaque

instability. This would occur by E2 increasing neovascularization around atherosclerotic plaques.

These contradictory effects of E2 are consistent with contradictory clinical results seen with hormone replacement therapy in post-menopausal women.

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VII. Appendices

Appendix 1

ing peroxynitrite, a potent oxidant which has further negative vascular affects [23]. The objective of this study was to determine if estrogen treatment of ovariectomized rats reduces vascular O_2^- production compared to untreated animals. Furthermore, we assessed the role of the newly described non-phagocytic NAD(P)H oxidase [24,25] in the production of O_2^- by examining the effect of diphenyleneiodonium (DPI), an inhibitor of flavin-containing enzymes and by adding NADH and NAD(P)H the substrates of this enzyme. NAD(P)H oxidase is a complex structure with multiple components. To be active, the components must assemble in the membrane. In phagocytic cells, p47phox is a key regulatory component [26]. We therefore also examined the expression of total and membrane associated p47phox. In addition, we determined if changes in O_2^- production are associated with evidence of increased bioavailablity of NO by examining the contractile and vasodilatory properties of rat aortic rings.

2. Methods

2.1. Animal preparation

All procedures were approved by the ethics subcommittee of the University Animal Care Committee. We used female Sprague-Dawley rats that were either intact or ovariectomized. Ovariectomy was performed by the supplier (Charles River Laboratories, St. Constant, QC) in 8-week-old animals (200–225 g) and shipped on the fifth day after surgery. A further 5–7 days were allowed for recovery. On day 14 after surgery, animals were anaesthetized with isoflurane and a hormone or placebo filled pellet (Innovative Research of America) was inserted subcutaneously in the neck. Pellets contained 0.25 mg of 17-β-estradiol (E2) or placebo for active product, which was released over 21 days. Animals were housed in pairs and allowed free access to food and water, in 12-h day-night regime.

After 21 days, rats were sacrificed by intraperitoneal injection of 80–100 mg of sodium pentobarbital (MTC Pharmaceuticals). Organs were quickly dissected, frozen in liquid nitrogen and stored at −80 °C for biochemical studies. For contraction studies, fresh 3 mm aortic rings were placed in 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. For the studies on vascular rings we also used male rats. These were larger (400–450 g) and older than the female rats. Male rats were not treated with hormones and the aortas were harvested as per the female rats.

2.1.1. Aorta rings bioassay

Vessels were cleaned and cut into ~3 mm segments and mounted horizontally between two stainless steel parallel hooks for the measurement of isometric tension (Grass-FT03 force transducer) and suspended in organ bath filled with 18 ml of Krebs-bicarbonate at 37 °C and pH 7.4. The chamber was continuously oxygenated with 95% O_2/5% CO_2. After equilibration, resting tension and maximal contraction in response to 60 mM of KCl were determined. The resting tension of the rat aorta was ~2 g. Dose–response curves to increasing doses of phenylephrine (10^{-9} to 10^{-4} M) were then obtained and the response expressed as a percentage of the maximal response to KCl. The integrity of the endothelium was assessed by testing the vasodilatory response of preconstricted rings to acetylcholine (Ach, of 10^{-10} to 10^{-4} M). The dose–response to phenylephrine was repeated in vessels in which the endothelium had been removed by gentle rubbing. Vessels were again preconstricted with 10^{-7} M phenylephrine and the vasodilatory response to SOD (1.5 μg/ml) or constriction with L-NAME (10^{-8} to 10^{-4} M) was tested.

2.1.2. Chemiluminescence

Vascular segments were placed in Krebs-HEPES buffer and incubated in the dark for 10 min at 37 °C. The tubes were transferred to a luminometer (Lumat LB 9501, Berthold Inc.) and 230 μM of lucigenin were added and the output of photons was measured for 7 min. The results with 230 μM were confirmed with 5 μM lucigenin to rule out artifacts from oxidative recycling of lucigenin [27]. The background signal was subtracted from the total output and the area under the curve of output versus time was calculated. We also measured the production of O_2^- by vessels incubated for 10 min at 37 °C with 100 μM of NADH or NADPH, the substrates of NAD(P)H oxidase. A standard curve was obtained by measuring the output of O_2^- in a spectrophotometer by the reduction of cytochrome C by xanthine/xanthine oxidase [28,29].

Sources of O_2^- production, were assessed by preincubating rings for 10 min with 300 μM diphenyleneiodonium (DPI), an inhibitor of flavin-containing enzymes, 300 μM of N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide synthases or 3 mM of tiron, a cell permeable non-enzymatic O_2^- chelator.

2.1.3. Immunoblotting

Tissues were obtained from rat hearts, spleen, diaphragm, lung and aorta, and homogenised in lysis buffer (HB) (in a ratio of 5 ml of buffer/gram tissue). The buffer had the following composition (pH 7.5): 50 mM Hepes, 150 mM sodium chloride (NaCl), 100 mM sodium fluoride (NaF), 10 mM tetrasodium pyrophosphate ($Na_4P_2O_7 \cdot 10H_2O$), 5 mM disodium ethylene diamine tetraacetate (EDTA·$2H_2O$), 0.5% Triton X-100, 0.5% deoxycholic acid ($C_{24}H_{39}O_4Na$), 5 mg/ml aprotinin, 2 mg/ml leupetin, 1 mg/ml pepstatin, 100 mM activated sodium orthovanadate (Na_3VO_4). Homogenates were centrifuged at 2000 × *g* for 30 min at 4 °C, the bottom pellet was discarded. The supernatant was resolved by SDS–PAGE and transferred to PVDF membranes. Protein was measured by the Bradford method. Membranes were incubated with monoclonal antibodies to p22phox, p47phox, p67phox (gift from Genentech), eNOS (Transduction Laboratories) and polyclonal gp91phox (gift from MR Quinn, Montana State University). For detection, membranes were incubated in chemiluminescence solution (Lumi-Light Plus, Roche) and visu-

alized by autoradiography (films Biomax-MR, Kodak). The blots were scanned with an imaging densitometer and optical densities of protein bands were quantified with SigmaGel software (Jandel Scientific Inc.).

2.1.4. Immunoprecipitation

The protein concentration of samples was adjusted to 1 mg per 0.5 ml with HB and then 5 μl of anti-p47phox monoclonal antibody (Genentech) was added and the mixture incubated for 12 h. Next we added 25 μl of protein A/G-agarose conjugate (Santa Cruz Biotechnology) for 3 h at 4 °C. Immunoprecipitates were pelleted by microcentrifugation at 10,000 rpm for 5 min. For electrophoresis, 15 μl of supernatant sample was added to 15 μl of loading buffer prepared from sample buffer (Invitrogen) with 5% β-mercaptoethanol. Pelleted beads were resuspended and washed with 0.5 ml of ice-cold immunoprecipitation HNTG-buffer with the following composition (pH 7.5): 20 mM Hepes, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, then microcentrifuged at 5000 rpm for 5 min. This supernatant was discarded after washing the beads. The pellet was washed in HNTG-buffer and centrifuged three times). After the final wash, the pellet was resuspended in 15 μl of electrophoresis loading buffer. All immunoprecipitation procedures were done on ice. Crude sample, pellet, and supernatant were resolved on SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membranes for immunostaining.

2.1.5. Preparation of membrane and cytosolic fractions

To determine cytosolic and membrane contents of p47phox, we added ice-cold buffer without deoxycholic acid to cardiac tissue and ground it in a blender until fully homogenized. The homogenate was centrifuged at 2000 × *g* for 20 min at 4 °C and the supernatant was saved. Nuclei and unbroken cells were centrifuged at 2900 × *g* for 15 min at 4 °C. Supernatant, which contains cytosol and membrane fractions, was ultra centrifuged at 29,000 × *g* for 45 min at 4 °C. Supernatant from this step was saved as a cytosolic fraction. The remaining pellet was resuspended and ultra centrifuged at 15000 × *g* for 20 min. The supernatant from this step was saved as the membrane fraction and run on SDS–PAGE and transferred to a PVDF membrane.

2.2. Radioimmunoassay for estrogen

Serum estradiol was measured with a competitive immunoassay using direct chemiluminescent technology. The assays were performed on the ACS/centaur automated analyzer (Chiron Diagnostics).

2.3. Statistics

Data are presented as mean ± S.E.M. Paired data were analyzed with a *t*-test. Multiple comparisons were performed with ANOVA and repeated measures with multiple conditions, as in the contractile studies, were analyzed with a two-way ANOVA for repeated measures. Statistical significance was accepted at $P < 0.05$.

3. Results

Estrogen treatment increased uterine weight from 0.14 ± 0.05 g in untreated animals to 0.57 ± 0.12 g in E2 treated animals. E2 treatment was associated with a decrease in body weight from 323.3 ± 19.5 g in control ovariectomized animals to 269.6 ± 0.12 g in E2 treated animal ($P < 0.001$). Plasma estradiol was 49 ± 18 pmol/l in untreated rats, which is in the range of post menopausal women, and 454 ± 185 pmol/l in rats treated with E2, which is in the range of the luteal phase of normal women [30] and therefore physiologic.

3.1. Superoxide production

Estrogen treatment lowered O_2^- production in rat aorta ($n = 12$ pairs, control and E2 treated) (Fig. 1a). The same pattern was seen with 5 μM of lucigenin as with 230 μM ($n = 3$ pairs) (Fig. 1a). The NAD(P)H inhibitor, DPI, decreased O_2^- production in untreated animals, but not in animals treated with E2 (Fig. 1b) whereas L-NAME had no effect on either group.

Incubation of rat aortic rings with NADH increased O_2^- production more than 100-fold, and there was no difference in E2 treated ($n = 8$) versus untreated rats ($n = 9$) (Fig. 2). NADPH also increased O_2^- production, but the increase was only a third of that seen with NADH, and again there was no

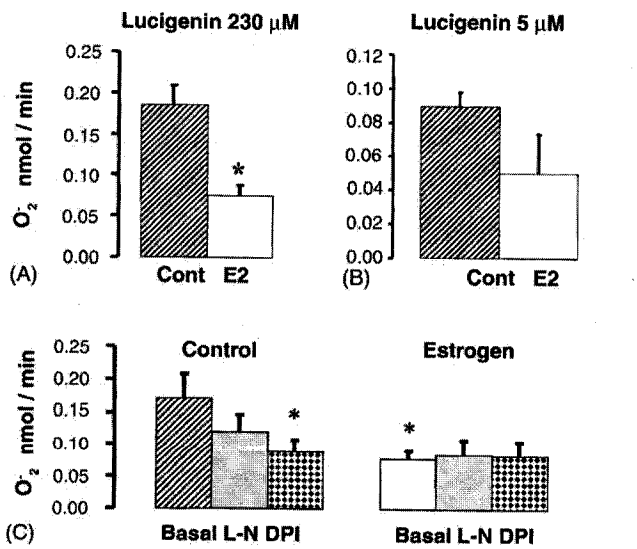

Fig. 1. Production of superoxide by aortic rings as determined by lucigenin enhanced chemiluminescence. (A) Production of O_2^- in nmol/min with 230 (M lucigenin ($n = 12$) (left) was significantly reduced. (B) The pattern was similar with 5 (M lucigenin ($n = 3$) Cont refers to control and E2 to 17-β-estradiol. (C) L-NAME (L-N) had no significant effect on O_2^- production in either control or E2 animals. DPI lowered O_2^- production to the same extent in control and E2 treated animals.

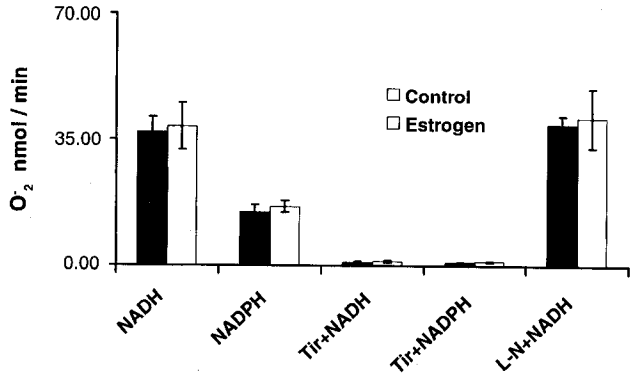

Fig. 2. NADH and NAD(P)H stimulated production of superoxide by aortic rings (control $n=9$, estrogen $n=8$). NADH markedly increased O_2^- production and so did NADPH, but to a smaller extent than NADH. Note the difference in scale from Fig. 1. Tiron almost completely inhibited O_2^- production produced by NADH and NADPH. L-NAME had no effect. There was no difference between control and E2 animals for any condition.

difference between treated and untreated rats. The O_2^- scavenger, tiron, markedly suppressed the increase in O_2^- seen with both NADH and NADPH. The nitric oxide inhibitor, L-NAME, had no effect.

3.2. Western analysis

There was no evident change in expression of p22phox in spleen and cardiac tissues. The p67phox component was studied in spleen, heart and lung and there was no evident regulation (data not shown).

Changes in p47phox were studied more extensively in cardiac tissue. E2 reduced overall expression of p47phox in crude samples (example in 3a) (density of 1147 ± 525 versus 568 ± 186 in estrogen treated, $n=10$, $P<0.05$). E2 decreased the expression of p47phox in the membrane fraction (Fig. 3b), but there was no evident change in the cytosolic fraction (Fig. 3c). In contrast to what we predicted, when we immunoprecipitated p47phox to concentrate the sample, more p47phox was immunoprecipitated in E2 treated than untreated animals (Fig. 4a), but there were only small differences in the supernatant (Fig. 4b).

3.3. Aortic ring studies

E2 did not change contractions induced by phenylephrine as a percentage of that produced by KCl (Fig. 5). We included untreated male rats ($n=9$) in this protocol; their curve was shifted to the left of that of female rats ($n=6$). Removing the endothelium did not alter the response, indicating that the difference in the response between males ($n=9$) and females ($n=6$) was not endothelial-dependent. Incubation of the rings with SOD produced the greatest dilation of partially constricted aortic rings in untreated females (untreated females, $n=6$; E2 treated females, $n=6$; males, $n=8$). SOD-induced dilation analyzed by groups was reduced after removal of the endothelium ($P<0.001$ by ANOVA for repeated measures), but differences between endothelial present or not present were only significant for male rats and oil-treated females by post hoc analysis ($P<0.001$).

The dose response to acetylcholine in pre-constricted aortic rings of male rats ($n=9$) was shifted to the left of the female rats ($n=6$), but there was no difference in treated or untreated females ($n=6$) (Fig. 6). There was greater constriction when L-NAME was added to partially constricted aortic rings in female rats compared to male rats, but again there were no differences between E2-treated and untreated rats.

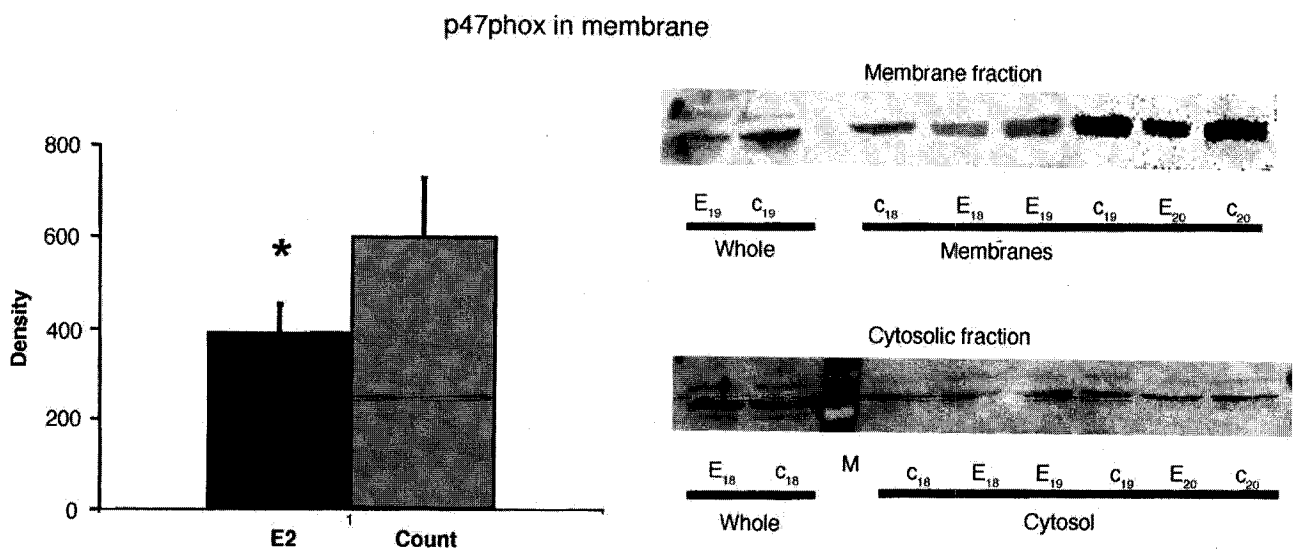

Fig. 3. Immunoblot of 47phox expression in hearts from ovariectomized rats treated with E2 or untreated. (A) Expression of p47phox in unfractionated tissue. Expression of p47phox was lower in E2 treated than in untreated animals (Cont) ($P<0.04$). (B) Examples of expression of p47phox in membrane fraction of cardiac tissue. Membrane expression of p47phox in treated rats (E) was less than in untreated rats (c). "Whole" refers to unfractionated samples. Animals were studied in pairs and the numbers refer to sample animal pairs. (C) Expression of p47phox in cytosolic fraction. There was no evident change in the cytosolic fraction with E2 treatment.

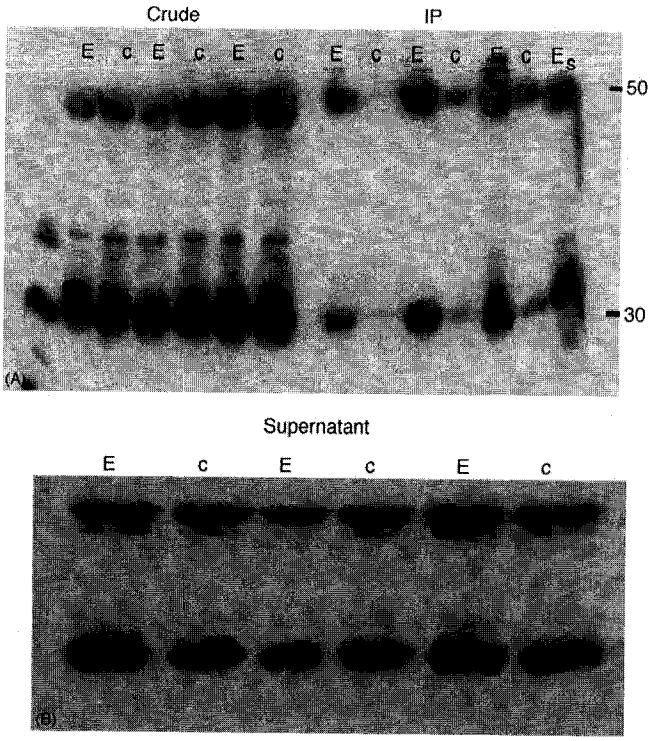

Fig. 4. Immunoblot of p47phox immunoprecipitated (IP) from cardiac tissue of ovariectomized rats. (A) Crude and IP samples from estrogen treated (E2) or untreated (c). Animals were treated in pairs and samples were placed on the gel accordingly. As in Fig. 3, E2 treated rats had lower p47phox expression in the crude sample of each pair. However, in contrast, p47phox expression was much lower in control rats compared to E2-treated rats. (B) p47phox expression in the supernatant from the IP. Expression of p47phox in c tended to be lower than E2 treated rats but difference was much smaller than in the IP fraction.

3.4. Endothelial NOS expression

By Western analysis, eNOS expression was actually lower in the aorta of E2 ($n=5$) treated compared to untreated rats ($n=5$) as well as intact female rats ($n=3$) ($P<0.05$) (Fig. 7).

4. Discussion

We found that O_2^- production was lower in the aorta of ovariectomized rats treated for three weeks with E2 than in untreated animals as has been observed recently by others [31,32]. We also found that DPI inhibited O_2^- production, which supports a role for NAD(P)H oxidase in the production of O_2^-. However, E2 did not alter NADH or NAD(P)H stimulated O_2^- production, which suggests that the amount of NAD(P)H oxidase did not change. The decrease in O_2^- production with E2 was associated with a decrease in the p47phox component of NAD(P)H oxidase, and in particular, the membrane component of p47phox, which supports the hypothesis that the regulatory effect of E2 occurs through alterations in p47phox. The decrease in O_2^- production was not associated with a change in the contractile response to phenylephrine or dilation in response to acetylcholine or SOD, which argues against an increase in bio-available NO. Finally, eNOS expression was not increased by the treatment and if anything decreased.

The production of reactive oxygen species, and O_2^- in particular, has been associated with vascular disease. In endothelial cells, increased O_2^- production decreases NO bioavailability, activates pro-atherosclerotic pathways and results in endothelial dysfunction [33,34]. A potentially important source of O_2^- production in vascular tissues is the recently described non-phagocytic form of NAD(P)H oxidase [36,39,40]. This enzyme complex is made up of at least six components, including p22phox, gp91phox, p67phox, p47phox, p40phox and small G proteins Rac and Rap1a and 2 and these components appear to be the same as those in phagocytic cell. The enzyme has been identified in aortic walls [28,29] and vascular smooth muscle cells [41] where its activation is involved in the vascular hypertrophy produced by angiotensin II [41–44]. It is also present in endothelial cells where its role is less clear [26,45,46].

We based our studies on the observation by Arnal et al. [15] who found that estrogen increased bio-available NO in endothelial cells without changing the expression of eNOS. They hypothesized that this could be due to decreased production of O_2^-, which would result in more bio-available NO, for it has long been known that O_2^- decreases bio-available NO [47,48]. They found that E2 decreased O_2^- production, but did not have a mechanism for this. In a subsequent publication [22], they showed that estrogen treatment decreases basal vascular O_2^- production. They also found that E2 shifted the aortic endothelial dependent vasodilation leftward, which is consistent with more available NO.

We hypothesized that estrogen reduces O_2^- production by decreasing the activity of NAD(P)H oxidase. Since activation of this complex requires the mobilization of p47phox and other cytosolic components to the membrane in phagocytic cells, we thus also hypothesized that regulation of p47phox is involved in the estrogen effect. In support of these hypotheses, basal O_2^- production was decreased and so was total p47phox expression and, more importantly, p47phox in the membrane.

Wagner et al. [45] also studied the effect of E2 on NAD(P)H oxidase in endothelial cells. They found that E2 did not change basal O_2^- production, but E2 decreased phorbol ester induced O_2^-production. Decreased O_2^- production was associated with decreased expression of adhesion molecules and chemokines in response to cyclic strain [45]. They proposed that the decreased O_2^- production was based on decreased expression of components of NAD(P)H oxidase including gp91phox, p22phox and p47phox. In contrast, we found no change in p22phox and p67phox expression in the two tissues examined and recently Gragasin et al. [49] found that E2 actually increased expression of gp91phox protein in bovine microvascular cells.

As already noted, in our study, total p47phox was decreased in the hearts of E2 treated rats compared to untreated animals, especially in the membrane component. This sug-

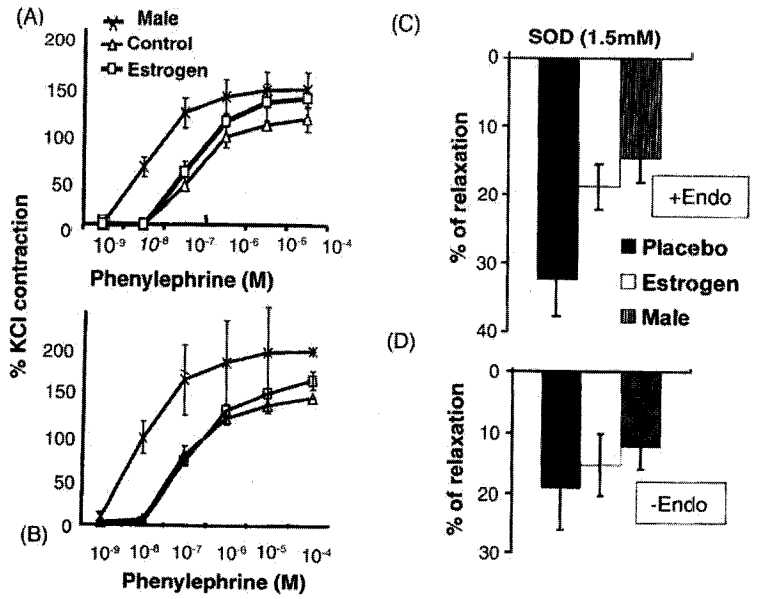

Fig. 5. Contractile responses to phenylepherine and relaxation with superoxide dismutase (SOD) in rat aorta. (A) Contractions with increasing doses of phenylepherine with the endothelium intact. (B) Contractions with the endothelium removed. Values were normalized to the contraction produced by KCl. The response in male rats (X) was shifted to the left of that of ovariectomized rats and reached a higher plateau. Treatment of ovariectomized rats with E2 (□) did not alter the contractile response compared to untreated rats (△) with or without the endothelium. (C) A bar graph of the superoxide dismutase (SOD) induced dilation of rings preconstricted with phenylepherine (10^{-7} M) with endothelium intact and in D with the endothelium removed. Untreated rats tended to have greater dilation than E2-treated or male rats but the difference was not significant. The group response in C (endothelium intact) was significantly greater than in D (endothelium removed) (by two-way ANOVA). By post hoc analysis, only the differences between control and males in the two conditions were significant.

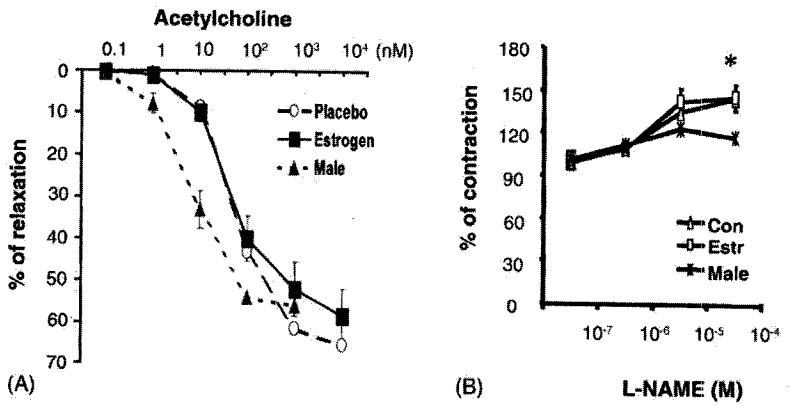

Fig. 6. Endothelial-dependent dilation and constriction of aortic rings from rats. (A) Ovariectomized rats had the same vasodilatory response to acetylcholine with (placebo, □) or without (O) treatment with E2. The response in male rats (△) was shifted to the left. (B) L-NAME produced greater vasoconstriction in ovariectomized rats than male rats (X) and the response was not difference in estrogen treated (□) or not treated (△).

gests that E2 reduces the attachment of p47phox to the membrane bound components, which is believed to be important for activation of the complex. A surprising finding was that despite there being a lower concentration of total p47phox, more p47phox was immunoprecipitated in E2-treated than untreated rats. This suggests that the epitope for the monoclonal antibody was less available in untreated animals, perhaps because more was attached to the NAD(P)H oxidase membrane complex. This could have occurred because of an E2 induced conformational change in p47phox when it is phosphorylated and activates the full NAD(P)H oxidase complex [50] Thus, even the reduction in total p47phox may not represent a true decrease but rather a decrease in the immunologically available protein.

It has recently been shown that decreased O_2^- production with E2 treatment in aorta of rats and vascular smooth muscle is associated with decreased Rac-1, a GTPase that is involved in the assembly of NAD(P)H oxidase [31]. This is thus an alternative or additional way that E2 could regulate NAD(P)H oxidase.

Stimulation of NAD(P)H oxidase by providing the substrates, NADH or NAD(P)H, did not alter O_2^- production in

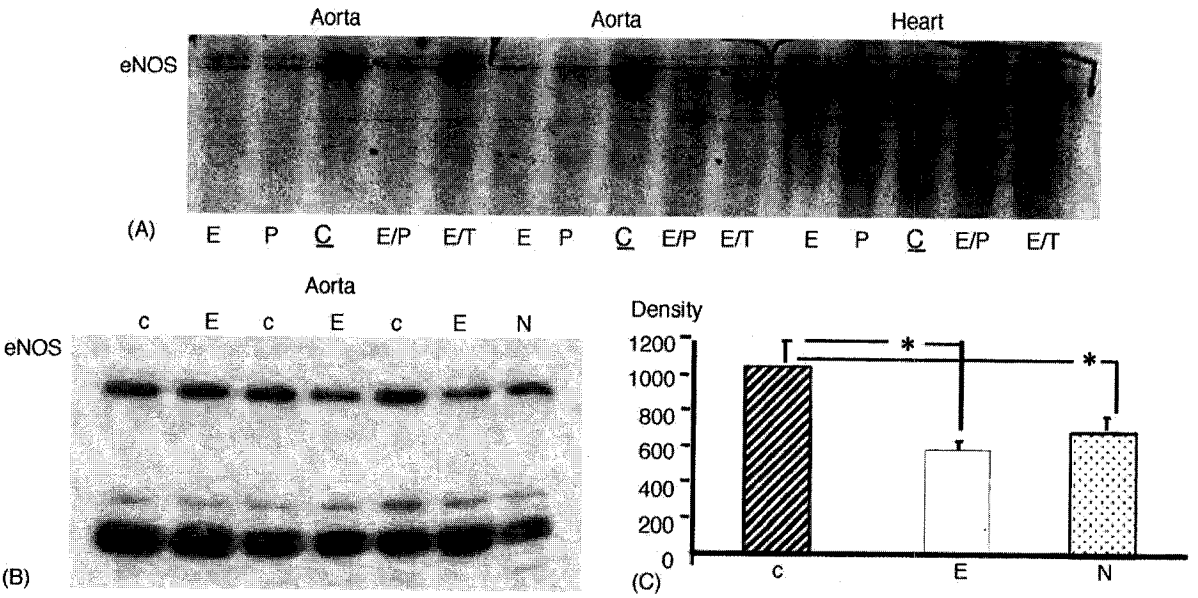

Fig. 7. Protein expression by western analysis in untreated aorta from ovariectomized rats (C), E2 treated ovariectomized rats (E) and intact female rats (W). Average densities are shown in the histogram. eNOS expression was higher untreated ovariectomized rat (c) ($n=5$) than in the E2-treated animals (E) ($n=5$) or intact females (N for normal) ($n=3$).

E2-treated compared to untreated rats. Since this stimulation tends to test the maximal capacity of the enzyme, it indirectly reflects the amount of enzyme present and the failure to see an E2 effect supports the argument that there was no significant change in expression of the components of the enzyme by E2 treatment of ovariectomised rats.

Wagner et al. [45] found that ovariectomy decreased eNOS expression, whereas we found no change in eNOS expression by Western analysis. Our findings are consistent with those of Arnal et al. [15,22]. Our studies on vascular rings also argue against a change in NOS expression or bioavailable NO. As also observed by Barbacanne et al. [22], E2 did not affect the contractile response to phenylephrine. However, in contrast to their study, we also found no difference in the vasodilatory response to acetylcholine. The contractile response to phenylephrine was greater in male rats than in female rats and was not affected by removal of the endothelium, which also argues against a change bio-available NO. The greater contractile response in males than in females could have been related to their larger size.

We expected that treatment of vascular rings with SOD could uncover a reduction of bio-available NO in untreated animals as observed by others [51], but the analysis is confounded by changes in both eNOS activity and O_2^- production. SOD treatment produced the greatest dilation in untreated ovariectomized females, consistent with a larger production of O_2^-. Removal of the endothelium greatly reduced the SOD induced dilatation in untreated rats, which supports an endothelial source for the O_2^-. Somewhat in contrast to our results, Hyashi et al. [51] found that intact female rabbits had greater dilation with SOD treatment than male rabbits or ovariectomized rabbits. Differences between our studies could be because our "intact" animals were treated with E2, and also because they used rabbits whereas we used rats.

The failure to see a leftward shift in the acetylcholine dose response curve (i.e. increased sensitivity) after E2 treatment at first seems to contradict a number of studies in which estrogen treated vessels from both animals and humans were studied [52,53]. We, too, previously have found a leftward shift of the acetylcholine dose response curve [21] and attributed it to activation of an Akt/PKB pathway as identified by others [20,54]. The difference may be that leftward shifts of the vasodilatory response have been demonstrated in acute studies, whereas in this study, animals were examined after three weeks of treatment. This may mean that compensatory mechanisms are activated over time.

A failure to find a difference in vascular responses in chronically treated versus untreated rats might be explained by production of endogenous estrogens in untreated animals. Ovariectomized rats gain weight after recovering from the surgery which means that there is more peripheral tissue which can make E2 by the conversion of adrenal androgen precursors to estradiol through the action of aromatase [55]. Indeed, we also found that the weight of untreated ovariectomized rats was higher than the weight of E2 treated rats. However, this cannot explain our results because E2 treated animals had almost ten times the estrogen concentration of untreated animals. An estrogen effect also was evident by the much higher uterine weights treated versus untreated animals.

5. Conclusions

Chronic estrogen treatment of ovariectomized rats decreased the production of O_2^- by the vascular wall, but this

was not associated with evidence of increased bio-available NO or a change in vascular contractile properties. The decrease in O_2^- production was associated with a decrease in the membrane fraction of p47phox, which suggests that estrogen treatment alters O_2^- production by regulating the association of p47phox with the full NAD(P)H oxidase complex. It is possible that the estrogen-induced decrease in O_2^- production could alter intracellular signaling and thereby contribute to the vascular protection observed with estrogen treatment.

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Appendix 2

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.