The reverse side also has a reverse side.

- Japanese proverb

From the Japanese Garden in Jardin Botanique de Montréal

University of Alberta

REGULATION OF VASCULAR FUNCTION BY ESTROGEN: IMPACT OF AGING

by

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DEDICATION

To my children;

Who taught me the most important life lessons.

ABSTRACT

Ovarian endocrine function has a major impact on homeostatic mechanisms in the cardiovascular system in women. In particular, estrogens play an important role in the regulation of vascular function. Epidemiological and experimental evidence links increased risk for cardiovascular disease with estrogen deficient states of various etiologies. It has however become recognized that vascular effects of exogenous estrogens are complex, and may result in a range of effects, from beneficial to contrabeneficial, depending on the vascular conditions. Although the mechanisms are not fully explained, vasoprotective potential of estrogen is attenuated in the presence of vascular risk factors, including aging (e.g., postmenopausal age). Aging is associated with pro-inflammatory and pro-oxidant alterations in the vascular microenvironment, along with an imbalance of local vasoactive mechanisms. The results of this thesis reinforce evidence of augmented endothelin (ET) mediated vasoconstriction together with deficient nitric oxide (NO) modulation of resistance artery function in an animal model of female aging (i.e. aging ovariectomized rat). We examined the hypothesis that dysfunction of the enzymes, matrix metalloproteinase (MMP) and neuronal nitric oxide synthase (nNOS), which are sources of ET and NO in the vasculature, contributes to the pro-hypertensive postmenopausal phenotype. We found that MMP played a significant role in vasoconstriction through generation of ET in aging, but not young females. On the other hand, in aging, there was a loss of nNOS-mediated vascular relaxation, where this enzyme further contributed to oxidative stress as a source of superoxide. Vasoprotective potential of estrogen was evaluated in both young and aging conditions, i.e. rats receiving estrogen replacement following ovariectomy. Our experimental findings

indicate that estrogen signaling is impaired in aging, partially as a consequence of dysfunction at the level of its mediators, MMP and nNOS. Indeed, estrogen treatment further increased the acute role of MMP in vasoconstriction and did not restore nNOS-mediated vasorelaxation. These studies illustrate the concept that fundamental vasoprotective pathways regulated by estrogen under controlled physiological conditions may turn dysfunctional in aging or other pro-inflammatory, pro-oxidant vascular states. Pharmacological interventions aimed selectively at these altered molecular pathways may yield more effective, non-hormonal therapeutic approaches in cardiovascular medicine.

PREFACE

Most of the work described in this thesis has been published or submitted for publication in excellent peer-reviewed journals. The work was done by Olga Lekontseva under the supervision of Dr. Sandra Davidge, unless otherwise noted. The full citations are listed below in order of their publication:

1) Chakrabarti S*, Lekontseva O*, Davidge ST. Estrogen is a modulator of vascular inflammation. IUBMB Life. 2008 Jun;60(6):376-82. Review, *equal contribution.

2) Lekontseva O, Jiang Y, Davidge ST. Estrogen replacement increases matrix metalloproteinase contribution to vasoconstriction in a rat model of menopause. J Hypertens. 2009 Aug;27(8):1602-8.

3) Lekontseva O*, Chakrabarti S*, Davidge ST. Endothelin in the female vasculature: a role in aging? Am J Physiol Regul Integr Comp Physiol. 2010 Mar;298(3):R509-16. Review, *equal contribution.

4) Lekontseva ON, Rueda-Clausen CF, Morton JS, Davidge ST. Ovariectomy in aged versus young rats augments matrix metalloproteinase-mediated vasoconstriction in mesenteric arteries. Menopause. 2010 May-Jun;17(3):516-23.

5) Lekontseva O, Chakrabarti S, Jiang Y, Cheung CC, Davidge ST. Role of neuronal nitric oxide synthase in estrogen-induced relaxation in rat resistance arteries. J Pharmacol Exp Ther. 2011 Nov;339(2):367-75.

6) Lekontseva O, Jiang Y, Schleppe C, Davidge ST. Altered neuronal nitric oxide synthase in the aging vascular system: implications for estrogen therapy. Endocrinology 2011 (in review).

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"I not only use all the brains that I have, but all that I can borrow"
- Dr. Thomas Woodrow Wilson

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LIST OF ABBREVIATIONS

ADMA……asymmetric dimethylarginine
AF-1, AF-2……activation function -1, -2
a.k.a……also known as
ANOVA……..analysis of variance
RMANOVA……...repeated measures analysis of variance
AP-1……activating protein -1
AUC……..area under the curve
BH_2……dihydrobiopterin
BH_4……...tetrahydrobiopterin
BK_{Ca}…….large-conductance calcium-activated potassium channels
Ca^{2+}…….....calcium ion
$CaCl_2$…….calcium chloride
CaM……calmodulin
cAMP……..cyclic adenosine monophosphate
cGMP……..cyclic guanidine monophosphate
CCRC……...cumulative concentration-response curve
-COOH……carboxy terminal
CRP……C-reactive protein
CV……cardiovascular
CVD……..cardiovascular disease
DAF-FM……..4-amino-5-methylamino-2,7-difluorofluorescein
DAPI……..4',6-diamidino-2-phenylindole
DBD……..DNA-binding domain
DHE……dihydroethdium
DMSO……dimethyl sulphoxide
DNA…… deoxyribonucleic acid
e^-……free electron
E2…….17-beta estradiol
EC_{50}, EC_{80}…….effective concentration eliciting 50%, 80% response
ECE…….endothelin-converting enzyme
ECGS……...endothelial cell growth supplement
ECM…….extracellular matrix

EDHF……endothelium-derived hyperpolarizing factor

EDTA……ethylenediaminetetraacetic acid

e.g……...*exempli gratia* (Latin, “for example”)

EGFR……...epidermal growth factor receptor

ER, ERα, ERβ……...estrogen receptor (alpha, beta)

ERE……..estrogen-response element

et al……..*et alii* (Latin, “and others”)

etc……*et cetera* (Latin, “and so on”)

ET (ET-1, ET-2, ET-3) ……endothelin (-1,-2,-3)

bET-1……..big endothelin-1

ppET-1……...preproendothelin-1

ETR, ET_AR, ET_BR……..endothelin receptor (A, B)

FAD……flavin adenine dinucleotide

Fe……..iron

FMN…….....flavin mononucleotide

FBS……...fetal bovine serum

FDA……..Food and Drug Administration

GPER……G protein-coupled estrogen receptor

GPR30……G protein-coupled receptor 30

HBSS……...Hank’s Balanced Salt Solution

HEPES…….hydroxyethyl-1-piperazineethanesulfonic acid

H_2O_2…….hydrogen peroxide

HSP90…….heat shock protein 90

HUVEC……..human umbilical vein endothelial cells

i.e……...*id est* (latin, “that is”)

IgG……...non-specific immunoglobulin G

IL-1β, IL-6……..interleukin-1beta, -6

K^+……..….potassium ion

KCl……potassium chloride

KH_2PO_4……..potassium dihydrogen phosphate

LBD…….ligand-binding domain

L-NAME…….....Nω-Nitro-L-arginine methyl ester

L-NPA…….N-propyl-L-arginine

L-VNIO……..N5-(1-imino-3-butenyl)-L-ornithine

MAPK……..mitogen-activated protein kinase

MCh……...methacholine

MFI……...mean fluorescence intensity

$MgSO_4$…….magnesium sulfate

MMP……..matrix metalloproteinase

mRNA……...messenger ribonucleic acid

MT-MMP……..membrane-type matrix metalloproteinase

NA…….noradrenaline

NaCl……..sodium chloride

NADPH…….nicotinamide adenine dinucleotide phosphate

NAN3…….sodium azide

NF-κB……nuclear factor kappa B

$-NH_2$…….amino terminal

NIH……National Institutes of Health

NO, NO•…….nitric oxide (radical)

NOS……..nitric oxide synthase

eNOS……..endothelial nitric oxide synthase

iNOS……...inducible nitric oxide synthase

nNOS……..neuronal nitric oxide synthase

ns…….....not significant

O_2……molecular oxygen

$O_2•^-$…….....superoxide anion

OH•…….....hydroxyl radical

$ONOO^-$…….peroxinitrite

Ovx……..ovariectomy, ovariectomized

OxLDL……oxidized low density lipoprotein

PBS……phosphate buffered saline

PGI_2……..prostacyclin

Phe……...phenylephrine

PI3K…….....phosphatidylinositol-3-kinase

PKA, PKC……...protein kinase A, C

PLC/ IP3…….....phospholipase C/ inositol triphosphate

PSS……..physiological saline solution

RNS……...reactive nitrogen species

ROS……reactive oxygen species
SDS……sodium dodecylsulphate
SERM……selective estrogen receptor modulator
SEM……..standard error of mean
sGC…….soluble guanylyl cyclase
-SH……..thiol group
SOD…….....superoxide dismutase
Sp1……..specificity protein 1
TIMP……...tissue inhibitor of matrix metalloproteinase
TNF……..tumor necrosis factor
Tris-HCl……..…tris(1)-amino methane hydrochloride
US……..United States
vs……versus
VSMC……vascular smooth muscle cells
v/v……volume per volume
vWF……..von Willebrand factor
WHI……..……Women's Health Initiative
Zn^{2+}……..zinc ion
$ZnCl_2$……zinc chloride

CHAPTER 1:

BACKGROUND TO THE DISSERTATION

1.1 INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death and disability, not only in men but also in women (contrary to popular belief). There is an increasing awareness that women experience CVD differently than men. Indeed, sex-related differences in both normal and pathological physiology of the heart and circulation are now characterized at various levels: from populations and subjects, extending into fundamental molecular processes.[1, 2] Despite impressive advances in cardiovascular (CV) medicine over the past few decades, dissimilarities in the susceptibility to CV risk factors and CVD pathogenesis in women (who, as a variable, had not been included in majority of experimental study designs)[3] continue to pose diagnostic and therapeutic challenges in this population. For example, we have come to understand that the major CV risk factors such as aging, diabetes, hypercholesterolemia and smoking have a greater impact in women with declining reproductive status. Furthermore, the initial attempts at replacing ovarian hormones (such as estrogen) in women of postmenopausal age raised many questions regarding the safety and efficacy of this approach to reduce CVD. Altogether, it unmasked our knowledge gap on the complexity of the vascular actions of estrogens, and emphasized the need in establishing solid scientific foundations to build future evidence-based practices. Thus, understanding the appropriate, female sex-specific use of preventive and treatment strategies has become an essential frontier in CV research. In recent years, the field has been strongly supported by a number of federal programs

throughout North America (http://www.thehearttruth.ca/ and http://www.goredforwomen.org/), as well as development of pharmacogenomics as a platform for personalized, sex-oriented medicine and growing public interest in “natural”, bioidentical hormones such as phytoestrogens.

The main research focus of this thesis is directed towards preclinical detection and understanding of the early mechanisms and markers for vascular dysfunction in female aging and ovarian deficiency. I will also examine direct vascular effects of estrogen in these conditions. To provide a background for this dissertation, I will first overview the basic biochemistry and pharmacology of estrogen, highlighting new insights that may help in the understanding of the complexities behind menopausal hormone therapies. I will then outline pathways regulated by estrogen in the vascular system focusing on the mechanisms of vascular reactivity. Emerging evidence suggesting possible contrabeneficial aspects of estrogen in certain conditions (e.g., presence of CV risk factors and/or co-morbidities) will be included briefly to provide a sufficient basis for subsequent discussion. Next, I will review the role of vascular aging, a confounding pro-inflammatory condition superimposed with ovarian deficiency in menopause. The mechanisms for age-related vascular dysfunction including a perturbed interplay between the two major endothelium-derived products, endothelin-1 and nitric oxide, will be included in this section. The physiology of two vascular enzymes involved in the respective biosynthetic pathways, matrix metalloproteinase (MMP) and nitric oxide synthase (NOS) will be overviewed. This background will lead to my hypothesis that MMPs are mediators of altered vasoactive mechanisms in

aging. On the other hand, neuronal type NOS recently described in vascular cells may contribute to vascular dysfunction in an estrogen-deficient state. Altogether, we hypothesized that estrogen signaling in the aging vascular system is impaired, in part due to its altered mediators, MMP and nNOS. These pathways, if understood, may yield pharmacological alternatives to hormone replacement, which would bypass unwanted, broad spectrum effects of estrogen in the vasculature and elsewhere.

1.2 ESTROGEN AND THE VASCULAR SYSTEM IN FEMALES

Estrogens play the key role in sexual and reproductive function. In addition, in both women and men, this hormone exerts a broad range of biological effects in the cardiovascular, immune, skeletal, and central nervous systems.

1.2.1 Estrogen Biosynthesis

The biosynthesis of sex steroids is well understood. Estrogen is a derivative of cholesterol, formed by reduction of the number of carbon atoms from 27 to 18 via intermediate steroid products. The key proximate precursors, androstenedione and testosterone, are converted to estrogens by the action of the enzyme aromatase. In women, estradiol (E2) is the principle bioactive circulating form of estrogen produced by the developing ovarian follicle. In other cases (e.g., in men or postmenopausal women) estrogens can be produced from the aromatization of androgens in peripheral tissues (e.g., adrenal glands, adipose tissue, blood vessels, etc.) or placenta in pregnant women. Estrogen metabolism occurs mainly in the liver and includes oxidation by cytochrome P450s, as well as conjugative reactions. Conjugates are then excreted via bile or urine, however certain metabolic products (e.g., hydroxy- and methoxyestrogens) still possess estrogenic activities.[4, 5] E2 metabolism varies depending on age and menopausal status, ethnic/genetic background, drug interactions and environmental factors (e.g., diet, smoking), as well as the route of exogenous administration, which all may influence the results of menopausal hormone

therapy. To note, aromatase is present in a number of non-reproductive tissues including the vascular system, which suggests the importance of local hormone synthesis in the regulation of target-organ function.[6] Moreover, only a minimal fraction of estrogens circulate free of carrier-proteins (sex hormone-binding globulins) and thus physiologically active. Not all the intricacies of local steroid metabolism in the vascular tissue are understood yet. However, with the growing therapeutic use of hormone metabolism inhibitors (e.g., aromatase inhibitors),[7] a better understanding of how the local balance between estrogens and androgens influences organ function in both sexes is essential, and is another frontier in CV research.[8-10]

1.2.2 Estrogen Receptors

Three types of estrogen receptors (ERs) are currently known. ERα and ERβ are the ligand-activated transcription factors, and GPER (a.k.a. GPR30), is a more recently described membrane G protein-coupled receptor.

1.2.2.1 Classical estrogen receptors. Both ERα and ERβ belong to the nuclear receptor family of transcription factors. They are encoded by individual genes with different chromosome locations, and vary in their distribution and expression within tissues, including the vascular tissue. This suggests functionally distinct roles of these receptors, although some functions may overlap.[11] Both ERs function to produce classical genomic effects, but may also act through non-genomic mechanisms (see section 1.2.3).

Complexity in the function of ER proteins has been recently appreciated, and may be due to epigenetic changes (e.g., methylation of the ER-encoding genes), multiple ER isoforms/splice variants, as well as posttranslational modifications of ER protein (e.g., phosphorylation, S-nitrosylation). For example, methylated genes for ERα and ERβ are associated with atherosclerotic tissue.[12, 13] As methylation increases with the passage of vascular cells in culture (a model for cellular senescence), it may be implicated in reduced ER expression or activity in the aging vasculature.[13]

Levels of ERα and ERβ in the vascular system are differentially regulated by estrogen itself.[14] This process appears to depend on a number of factors, including the type of the vascular bed or cultured cells, duration of estrogen exposure and co-existing conditions (e.g., inflammation, hyperglycemia, etc). In a recent report, 24-hour exposure of endothelial cells to E2 led to selective increase in ERα relative to ERβ under normal conditions. In contrast, a stressful inflammatory state reversed the E2 effect on endothelial ER expression, i.e. decreased ERα to ERβ ratio.[15] Further understanding of the ligand-dependent regulation of vascular ERs may have major implications for interpretation of hormone treatment studies in humans.

Interestingly, other hormones and growth factors can activate ERs. For example, growth factors tyrosine kinases can phosphorylate and activate ER in the absence of the cognate ligand, estrogen.[16, 17] Progesterone can also affect levels of ERs. In particular, progesterone receptor A has been shown to function as a ligand-dependent trans-repressor of other steroid receptors, including ER.[18]

The physiological implications of the estrogen-independent regulation of ER activity have not been clarified beyond its role in female cancers, yet may also contain answers to understanding CV consequences of combined hormone replacement regimens.

ERα gene polymorphisms have been described in association with the risk of atherosclerosis and myocardial infarction in women and men.[19, 20] However, this is an emerging field and more work is needed to explore the link between ER polymorphisms and CVD. If understood, a potential application in the screening of candidates for hormone replacement therapy may emerge.

1.2.2.2 G protein-coupled estrogen receptor. There is a long history of observations demonstrating that estrogen also acts via plasma membrane receptors. One such receptor, a member of G protein-coupled receptors family, GPR30, has been shown to specifically bind estrogen, leading to activation of second messengers and a rapid cellular response.[21] To date, most of GPR30 functions have been studied in various tumor cell lines, whereas its role in mediating estrogen actions in the vascular system (where it is also present)[22] awaits further understanding. Interestingly, recent evidence supports GPR30 role in regulating estrogen-dependent vascular tone and blood pressure (described in section 1.2.3.2). As discussed in the following section, there are in fact a number of mechanisms to account for rapid non-genomic vascular actions of estrogen.

1.2.3 General Mechanisms of Estrogen Action

Both classical subtypes of ER contain several domains that are well conserved across the species.[23] These include the ligand-binding domain (LBD),

the DNA-binding domain (DBD), and two distinct activation functions domains (AF-1, and -2) which facilitate recruitment of co-regulatory proteins to the DNA-bound ER. Both ERα and ERβ have similar affinities for E2 and recognize the same hormone response elements in the DNA due to homology in their LBD and DBD. However, the two receptors vary greatly in the AF transcription activation sites. A schematic structure of ER is illustrated in Figure 1-1.

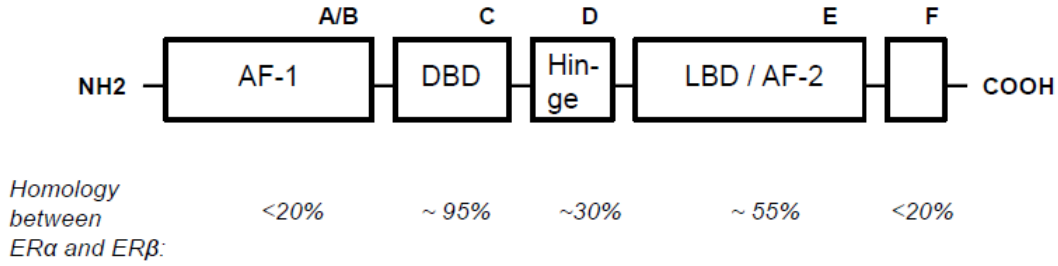

Figure 1-1. Schematic structure of a classical ER

Functional domains are illustrated with boxes. The relative size of each domain and the amino acid homology between the receptor subtypes is indicated. A/B domain contains AF-1 transcription activation site, and in an inactive state associates with chaperone proteins. C domain is a DNA-binding domain. D is a hinge region involved in the process of ER dimerization and nuclear translocation. E is a ligand-binding domain, which also contains AF-2 site involved in co-activator/co-repressor binding. F domain plays role in specific conformational change of the ligand-bound ER that defines its agonist or antagonist properties.

Ligand-dependent estrogen signaling begins with the binding of the free hormone (as it diffuses through the cell membrane) to ER. Thereafter, cell-specific response to estrogen may follow different molecular pathways.

1.2.3.1 Genomic effects. According to the classical genomic course of estrogen signaling, ligand-bound ER undergoes a conformational change that leads to dissociation from chaperones (e.g., heat shock proteins), dimerization and translocation to the nucleus. Thereafter, the ER complex can either directly bind to estrogen response elements (ERE) in the promoters of target genes or interact with other transcription factor complexes such as AP-1, Sp1 or NF-κB to influence transcription of genes whose promoters do not carry EREs. Subsequently, specific co-activator and/or co-repressor proteins are recruited to the ER-transcription complex, which collectively enables fine regulation of the transcriptional activity (Figure 1-2).[23] Variation in the co-regulator proteins between tissues is another contributor to the specificity of estrogen actions. Various ligands can induce different conformations of ERs, which may lead to the recruitment of a distinct set of co-regulators to the transcription complex, allowing for highly specific effects.[24] A successfully designed new class of drugs, selective estrogen receptor modulators (SERMs), can serve an example of tissue-selective ER agonism or antagonism of the same ligand. Although the mechanisms for such selectivity are complex and only partially understood, it is thought that a bulky side chain in the SERM molecules cannot be contained in the ligand-binding pocket, and impedes on the nearby AF-2 site.[25] This would interfere with AF-2 but not AF-1 function, which is located at the N-terminus of ER (Figure 1-1).

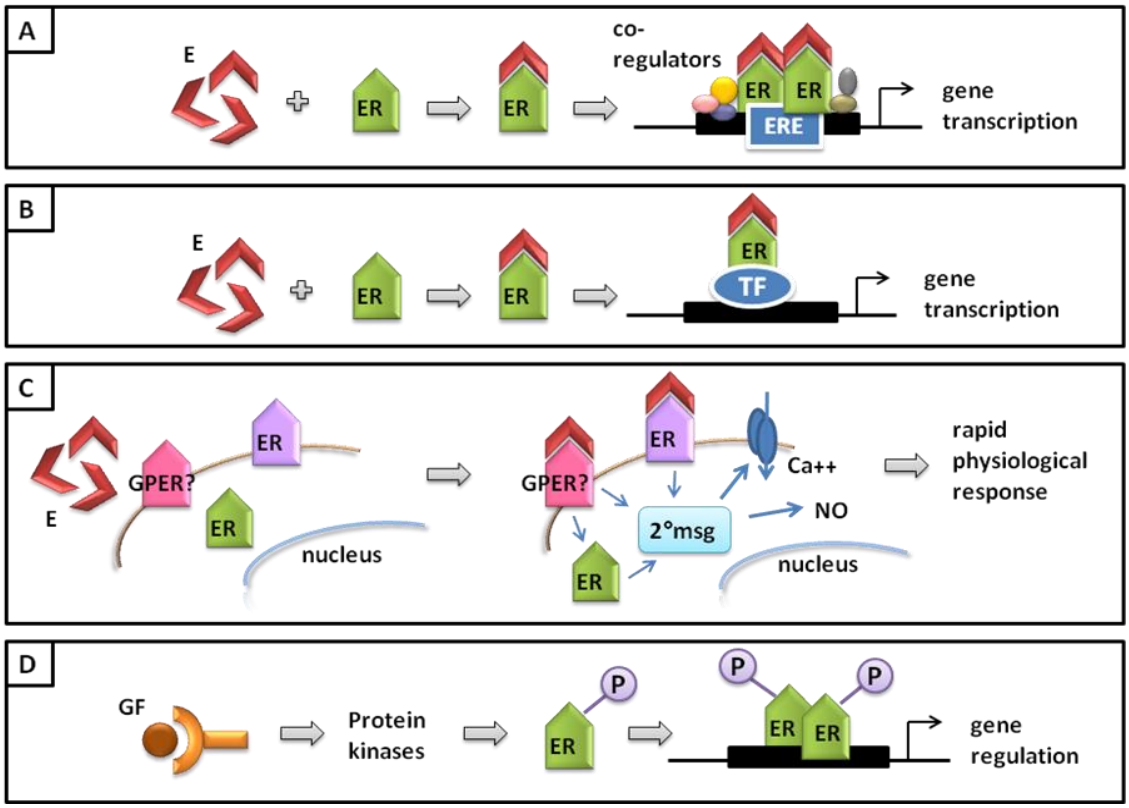

Figure 1-2. Basic signaling pathways initiated via ERs
Modified from Heldring et al. 2007 Physiological Reviews[26]

A. The classical ligand-dependent pathway involves estrogen (E) activation of estrogen receptor (ER), its dimerization and DNA binding via estrogen-response elements (ERE), followed by recruitment of specific co-regulatory proteins to modulate gene expression.

B. The tethered pathway involves protein-protein interactions of E-activated ER with other transcription factors (TF) (e.g., AP-1 or Sp1), and indirect DNA binding.

C. The non-genomic pathway is less understood: E activates a receptor associated with the cell membrane, or a signal activates the classical cytoplasmic ER. Downstream second messengers (2°msg) affect ion channels or increase nitric oxide (NO) levels in the cytoplasm, translating into a rapid cellular response.

D. The ligand-independent pathway includes ER activation through other signals such as growth factors (GF). Downstream kinases phosphorylate ERs to dimerize and bind DNA.

1.2.3.2 Non-genomic effects. In addition to the well-studied effects on gene transcription, estrogen is known to mediate non-nuclear effects via rapid activation of signaling kinases (such as phosphatidylinositol 3-kinase (PI3K)/Akt, mitogen-activated protein kinases (MAPK), protein kinase C (PKC)), generation of second messengers (like nitric oxide and cyclic amines, cAMP and cGMP), and activation of ion fluxes (e.g., calcium). Unlike the classical genomic effects, non-genomic actions can be mediated by ERs localized to the cell membrane, and can explain rapid effects of estrogen in tissues, including the vascular sytem (Figure 1-2).[27] Various terms, such as “rapid” or “non-genomic”, have been used to distinguish the cell surface initiated estrogen signaling from the transcriptional (i.e. nuclear-initiated) events. This distinction, however, remains arbitrary, since activation of intracellular signaling pathways can also impact longer term gene expression via various mechanisms (e.g., phosphorylation and activation of transcription factors by MAPK).

The nature of the cell surface ER has been debatable. In vascular endothelial cells, predominantly ERα subtype, both full-length and truncated forms, have been described in association with caveolae.[28] A number of other signaling molecules (e.g., G proteins, Src kinases, caveolin-1, etc.) congregate in a confined space in caveolae facilitating signal transduction. Although less well characterized, a functional ERβ has also been found at the membrane of endothelial cells.[29] Beside the classical ERs at the cell membrane, GPER (GPR30) is a structurally unrelated transmembrane receptor mediating estrogen actions via activation of G proteins. The initiated signaling cascades, including

kinase activation and calcium signaling can similarly lead to rapid, as well as long-term biological outcomes. Many groups have described alliance of ERα and GPR30 in a functional complex (rather than GPR30 as a stand-alone receptor for estrogen).[30] In fact, complex cellular pathways regulated by estrogen via GPR30 are better understood in cancer cell lines. Although, the functional role of GPR30 in the vasculature remains to be delineated, a striking similarity between certain fundamental pro-hypertensive and pro-carcinogenic pathways has been found. Of particular interest, GPR30-mediated matrix metalloproteinase (MMP) activation as a mechanism of cancer cell growth appears to recapitulate in the pathogenesis of hypertensive vascular disease.[31] Although in the latter case other G protein-coupled receptors have been directly examined (such as vascular adrenoceptors), MMP activation was proposed as a common mechanism downstream of G proteins, regardless of the nature of the ligand.[31] Another recent study indicates that chronic administration of selective GPR30 agonist G-1 lowers blood pressure in ovariectomized (Ovx) mRen2.Lewis rats, an estrogen-dependent model of hypertension.[32] Acutely, G-1 leads to relaxation of isolated rodent and human blood vessels, which is minimal in GPER-/- mice.[33-35] A more detailed summary of favorable CV actions of estrogen follows with the next section.

1.3 VASOPROTECTIVE ROLE OF ESTROGEN

The CV protective potential of estrogen has long been suspected, given the lower incidence of CVD (such as hypertension and coronary artery disease) in adult premenopausal women relative to age-matched men or women after menopause. These epidemiological data have been originally derived from the Framingham cohort assembled in 1948, which later turned out to be an ambitious project spanning several generations (www.framinghamheartstudy.org). Subsequently, evidence supporting CV benefits of postmenopausal estrogen replacement had been gathered through retrospective and observational clinical studies (e.g., Nurses' Health Study),[36] including meta-analyses of these studies. Although this has recently come into question based on large-scale randomized clinical trials (section 1.3.3), the favorable biological effects of estrogen in the CV system have been ascertained in a variety of experimental models. Indeed, in the vasculature, estrogen regulates a number of mechanisms that either indirectly, or via direct signaling in the vascular cells (both genomic and non-genomic) are protective to vascular structure and function.

An indirect beneficial role of estrogen is attributed to improving lipid profile, including resistance of lipoprotein particles to oxidation. This generally favors an anti-atherogenic vascular phenotype, and is largely a result of ER-mediated modulation of the hepatic gene expression. Additionally, estrogen stimulates peripheral adipose lipid metabolism and energy expenditure, while reducing

energy intake via central mechanisms, i.e. net “anti-metabolic syndrome” effects.[37]

Direct effects of estrogen on vascular homeostasis extend to modulation of vascular tone via multiple mechanisms, including circulating and local vasoactive factors, intrinsic myogenic mechanisms, and the autonomic nervous input. In the next section, I will outline key vasoactive pathways that are modulated by estrogen in the endothelial and vascular smooth muscle cells (VSMC).

1.3.1 Estrogen and Vascular Reactivity

The three most significant vasodilators produced by the vascular endothelium: nitric oxide (NO), prostacyclin (PGI_2) and endothelium-derived hyperpolarizing factor (EDHF) play key roles in regulating underlying VSMC contractility. Enhanced endothelial function in females (compared to males) can be exemplified by a greater brachial artery flow-mediated dilation, where the difference is more pronounced during the highest levels of endogenous E2 in the menstrual cycle and abolished during the lowest E2.[38]

One of the best described vascular actions of estrogen is increasing bioavailable NO in the endothelial cells, which ensures NO-mediated vasodilator responses. There is a considerable sexual dimorphism in NO production, which positively correlates with estrogen levels: for example, greater NO release was measured in aortic rings from female compared to male rabbits,[39] NO production was further increased during pregnancy[40] and during the preovulatory phase of the menstrual cycle.[41] In the long term, estrogen is known to upregulate NO

synthase (NOS) in the endothelium, classically via ERα.[42] Additionally, estrogen causes rapid activation of endothelial NOS (eNOS) via PI3K/Akt pathway initiated primarily from ERα associated with caveolae.[43] Yet another major mechanism, whereby estrogen maintains NO available for vascular relaxation, is via reducing oxidative stress (section 1.3.2) and thus preventing NO consumption in its reaction with free radicals. Evidence of the importance of estrogen for eNOS function continues to grow and recently includes new mechanisms, such as increasing endothelial availability of tetrahydrobiopterin (BH_4), an essential cofactor for NO synthesis,[44] and reducing endothelial production of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NOS.[45]

It has been demonstrated that estrogen has positive effects on other endothelial vasodilators, including the aforementioned PGI_2 and EDHF. For example, longer term exposure to estrogen increases cyclooxygenase-1 and prostacyclin synthase expression, favouring generation of vasodilator prostanoids.[46] The EDHF pathway predominantly ensures endothelial modulation of VSMC tone in small arteries, and may act as a backup endothelial vasodilator, when NO production is compromised. Several studies suggest a greater EDHF-compensation after NO inhibition in female arteries compared to male or Ovx female rats, where it can be enhanced by E2 administration.[47] One mechanism whereby estrogen deficiency can impair EDHF-mediated vasorelaxation, is by downregulating myoendothelial gap junction proteins such as connexins.[48] However, this is a relatively new field and much remains to be understood regarding the nature of EDHF itself and the specific EDHF component regulated

by estrogen in various vascular beds, which is beyond the scope of this thesis.

Besides the endothelium-dependent mechanisms, estrogen regulates vascular reactivity via the mechanisms inherent to VSMC. VSMC contraction is triggered by an increase in intracellular calcium, subsequent activation of myosin light chain kinase and myosin light chain phosphorylation that initiate actin-myosin cross-bridging. PKC, MAPK and Rho-kinase activity contribute to contractile mechanisms by modulating myofilament function and sensitivity to calcium. Experimentally, E2 can attenuate VSMC contraction through activation of BK_{Ca} potassium channels leading to increased K^+ efflux and membrane hyperpolarization.[49-51] Inhibition of calcium entry via voltage-gated calcium channels is another mechanism whereby E2 can elicit vascular relaxation, even in the absence of endothelium.[52] Furthermore, expression/activity of the vascular PKC isoforms and Rho-kinase (both of which have been implicated in the pathogenesis of CVD) can be reduced by estrogen in a variety of models.[53, 54]

Besides the intracellular events, estrogen regulates VSMC receptors for a number of agonists, including sympathetic mediators and vasoconstrictor peptides. For example, estrogen deficient states are associated with increased contribution of angiotensin and endothelin (ET) system to vascular dysfunction, reversible by E2 treatment.[55, 56] In particular, E2 reduces endothelial release of ET and its VSMC receptor expression.[57-59] In addition, E2 reduces norepinephrine discharge and sympathetic tone,[60] for example, by interfering with catecholamine synthesis[61] and sympathetic nerve growth.[62]

In summary, estrogen antagonizes vasoconstrictor pathways (at least in

healthy conditions) via modulating the balance of vasoactive mediators, supporting endothelial function and ameliorating VSMC reactivity.

In addition, estrogen-dependent anti-oxidant, anti-inflammatory, and anti-proliferative pathways in vascular cells protect against injury from exposure to risk factors, such as elevated blood pressure, circulating lipids and/or glucose, etc. In brief, I will present evidence for the recently recognized anti-inflammatory properties of estrogen in the vascular system.

1.3.2 Anti-inflammatory Role of Estrogen*

Estrogen is a modulator of vascular inflammation. Its anti-atherosclerotic, anti-inflammatory effects span numerous vascular pathways, including NO generation, suppression of cytokines and reduction of oxidative stress.

NO is an important vasoprotective molecule, whose role extends far beyond its vasodilatory activity. In fact, NO regulates a wide range of anti-inflammatory mechanisms that prevent endothelial cell activation, impede leukocyte recruitment and reduce reactive oxygen species (ROS). Therefore, a part of the anti-inflammatory potential of estrogen can be attributed to increasing NO bioavailability as previously discussed (section 1.3.1).

Oxidative stress in the vasculature is a major cause of vascular cell damage under inflammatory CVD conditions. It is caused by raised intracellular levels of ROS such as superoxide ($O_2\bullet^-$). Superoxide anions are normally

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* *Modified from the review: S Chakrabarti, O Lekontseva, ST Davidge. IUBMB Life 2008; 60(6):376-82.*

converted to a less harmful hydrogen peroxide (H_2O_2) by the enzyme superoxide dismutase (SOD). Increased levels of superoxide in vascular cells can react with (and consume) endothelial NO to generate peroxynitrite ($ONOO^-$), which itself is a potent pro-oxidant perpetuating a vicious cycle of inflammation and oxidative stress. Estrogen acts as an antioxidant by upregulating SOD in the vascular tissue, which improves clearance of superoxide anions.[63] In addition to this genomic effect, owing to its phenolic structure, estrogen can chemically bind and detoxify the superoxide anions.[64] Interestingly, many structurally similar dietary polyphenol compounds (e.g., derived from red grapes wine) mediate their CV protective actions partly via ER-dependent mechanisms.[65] Thus, estrogen has the capacity to attenuate inflammation through its antioxidant properties.

Another important aspect of the anti-inflammatory properties of estrogen is its interaction with immune factors. Estrogen suppresses the synthesis and signaling of pro-inflammatory cytokines (such as tumor necrosis factor (TNF), angiotensin II and ET), which prevents downstream alterations in vascular structure and function.[66] In particular, elevated TNF has been implicated in the pathogenesis of CVD.[67] Cytokine-activated endothelial cells express adhesion molecules and chemotactic factors that facilitate recruitment of immune cells into the intima. Furthermore, TNF increases oxidative stress through activation of NADPH oxidase and downregulation of eNOS. Resulting endothelial dysfunction with impaired endothelium-dependent relaxation leads to a compromise in vessel tone regulation. TNF-stimulated VSMC dedifferentiate to a pro-synthetic phenotype, and are prone to proliferation and excessive extracellular matrix

deposition contributing to structural remodelling. Elevated TNF levels are associated with estrogen-deficient states in animal models and humans,[68, 69] whereas estrogen counteracts TNF-mediated vascular dysfunction in different experimental settings.[66] For example, E2 acting via ERα attenuates TNF effects through NF-κB inhibition and consequent reduction in adhesion molecule expression and leukocyte transmigration across the endothelium.[70] Another study, found ERβ-mediated attenuation of TNF-induced VSMC expression of pro-inflammatory mediators and neutrophil chemotactic activity.[71] Although some understanding has been achieved, the mechanisms of cytokine-induced vascular dysfunction in estrogen deficiency are still the subject of investigations.

In summary, multiple protective pathways regulated by estrogen in the vascular system, in a variety of *in vivo* and *in vitro* models, contribute to prevention and/or dampening of inflammation. This was regarded as a plausible explanation for the low-risk CV profile in adult women, which might hypothetically be reconstituted through the hormone replacement after menopause.

1.3.3 Estrogen Replacement: Molecular Complexity behind Clinical Simplicity

"All things are difficult before they are easy"

- Dr. Thomas Fuller, Gnomologia (1732)

The landmark large-scale randomized clinical trials of postmenopausal hormone replacement such as the Women's Health Initiative (WHI)[72] and the

Heart and Estrogen/Progestin Replacement Study (HERS)[73, 74] challenged hormone research over the past decade. Possible reasons for the conflict between the known beneficial vascular role of estrogen and the neutral to detrimental results of these trials have been widely discussed in the literature.[75] Some of the findings were critiqued due to study design and statistical analysis. For example, highly debated aspects included the participant's age, timing of the therapy and the formulation of estrogen,[76] the route of administration and combination with progestin,[77, 78] pre-existing CVD or risk factors and socioeconomic status, as well as calculation of relative versus absolute risks for outcomes in WHI.[79] The lessons learned subsequently led to more directed trials such as the recent Kronos Early Estrogen Prevention Study (KEEPS)[80] and the Early Versus Late Intervention Trial with Estradiol (ELITE),[81] as well as careful design of laboratory models. Many researchers have converged on the "timing hypothesis", which postulates that estrogen signaling pathways are impaired in older women due to subclinical vascular damage. In the setting of altered vascular background, the anti-inflammatory vasoprotective mechanisms may no longer function, whereas estrogen itself may promote inflammation. Altogether, clinical studies have raised many questions that at present cannot be easily answered in human subjects and require in-depth fundamental insights. In the next section, I will highlight some publications that provide new mechanistic insights on how estrogen exposure can further aggravate pre-existing vascular compromise such as that induced by common CV risk factors like hyperglycemia, hyperlipidemia and aging.

1.3.3.1 Estrogen in the presence of cardiovascular risk factors.[*]

Estrogen and Hyperglycemia. The study by Hao-Liang Xu et al. demonstrated that E2 replacement in Ovx diabetic rats potentiated post-ischemic brain injury in a stroke model, as measured by leukocyte adhesion and extravasation, hallmarks of inflammation.[82] In contrast, this group previously reported protective effects of E2 in a similar model of stroke in non-diabetic rats, and related this to NF-κB inhibition and reduced adhesion molecule expression in the cerebral endothelial cells.[83, 84] Mechanisms underlying impaired estrogen signaling under hyperglycemic conditions are just beginning to be elucidated, and may explain increased vascular complications in women with diabetes.

Estrogen and Hypercholesterolemia. Umetani et al. suggested that estrogen signaling could be inhibited by the cholesterol metabolite, 27-hydroxycholesterol (27-HC), which is elevated in hypercholesterolemia and found in atherosclerotic lesions. 27-HC, acting as an endogenous selective inhibitor of vascular ERs, reduces estrogen-dependent NO production, resulting in diminished vascular relaxation.[85] This might explain the loss of estrogen-mediated vasoprotection in women with increased cholesterol levels.

Estrogen and Oxidative Stress. Powerful antioxidant properties of estrogen have, in part, been attributed to eNOS activation. However, eNOS plays an important role in the production of both NO and $O_2^{\bullet -}$. Alterations in the availability of cofactors for NO synthesis under pathological conditions may lead to eNOS

[*] *Modified from the review: S Chakrabarti, O Lekontseva, ST Davidge. IUBMB Life 2008; 60(6):376-82.*

uncoupling (further discussed in 1.6.2.2), which would shift the enzyme complex to superoxide production.[86] In this case, estrogen stimulation of uncoupled eNOS may increase superoxide generation, further reinforcing the vicious cycle of inflammation. On the other hand, in situation where eNOS is not uncoupled, estrogen stimulation can provide more NO to react with superoxide, yielding the powerful nitrating and inflammatory molecule, peroxynitrite.[87] Thus, the damaging effects of estrogen in vascular disorders associated with oxidative stress could result from peroxynitrite-induced protein nitration resulting in a loss of important cellular functions or induction of NF-κB, a global inflammatory transcription factor, as well as reduced bioavailability of NO, a natural anti-inflammatory molecule.

Estrogen and Aging. Attenuation of vasoprotection has recently been shown in E2 supplementation experiments in aging animals and postmenopausal women.[88, 89] In the study by Miller et al., carotid artery response to balloon injury was measured in young Ovx and aged Ovx rats, which were treated with either placebo or E2. E2 attenuated neointima formation and expression of pro-inflammatory mediators in injured arteries from young rats (compared to placebo group), however it failed to reduce inflammatory response in aged E2-treated rats (versus placebo).[88] Similarly, Sherwood et al. found a favorable effect of short-term transdermal E2 on brachial artery flow-mediated dilation in 50-59 years old women, but not 60-69 or 70-79 years old.[89] The mechanisms explaining the loss (or reversal) of vasoprotective potential of estrogen in aging are not clear, but hypothetically related to oxidative and inflammatory overstimulation in the vascular tissue. A study by Pereira et al. demonstrated increased plasma levels

of nitrotyrosine together with decreased nitrosothiols in postmenopausal women.[90] Whereas nitrotyrosine is the key marker of peroxidation processes, formation of nitrosothiols (via S-nitrosylation of cysteine residues) is considered a reversible and favorable protein modification, as well as an NO reservoir. Interestingly, activity of ERs can also be modulated by the action of oxidizing agents at the exposed Cys residues that coordinate zinc fingers in the DNA-binding domain.[91] For example, NO-induced S-nitrosylation of ERα inhibits its DNA-binding function (i.e. genomic effects) that may favor rapid non-genomic signaling via ERα.[92, 93] However, oxidative stress in the aging vascular tissue may impair this protective mechanism. Furthermore, basal levels of inflammatory factors, like TNF, significantly increase in healthy postmenopausal women, and may also contribute to altered effects of estrogen in the aging vasculature.[94] For example, in cultured human endothelial cells stimulated with TNF, E2 further increased cell surface expression of adhesion molecules and leukocyte binding.[95]

In summary, the net *in vivo* vascular effects of exogenous estrogen appear more complex than the direct fundamental pathways regulated in the vascular cells. Although the pathogenesis of vascular dysfunction in postmenopausal women is not completely understood, it is believed to result from a complex interplay between the decline in ovarian hormones and the aging process. Understanding this interplay of mechanisms may help in designing stratified and more individual hormone treatments, or possibly, substitutes for conventional hormone therapies. The nature of aging, as one of the major confounding factors in hormone replacement, will be discussed in a more detail in the next section.

1.4 AGING AND CHANGES IN VASCULAR FUNCTION

There is now a critical mass of data to support the hypothesis that the failure to replicate the vascular benefits of estrogen in clinical settings could be due to the aging process. Indeed, age-related changes develop in every organ system, including endocrine, immune and vascular senescence. For example, the endocrine aspect of aging includes a natural decline in sex hormone levels and metabolism, ER amount, distribution and integrity, as well as post-ER signaling pathways (selective decline of non-genomic vs. genomic).[96] Age-related changes in other sex hormones such as progesterone and testosterone alter the hormonal environment that likely influences the CV effects of estrogen. Immune senescence has been tightly linked to the endocrine decline, and is overall characterized by deterioration of the host defense mechanisms, including overproduction of pro-inflammatory cytokines.[97] While short-term hormone replacement studies in early postmenopausal women suggest improvement in many immune parameters (including suppression of cytokines),[98] there seem to be more complex effects on the cytokine profile seen in older women.[99] A chronic subclinical pro-inflammatory state may further augment vascular aging that involves remodelling processes, which overtime may propagate to overt CVD. In fact, aging is the strongest, independent predictor for the CV events, which is known to promote CVD even in the absence (or adequate control) of the other major risk factors. However, early markers and molecular events in the vascular aging, if understood, may present a window of opportunity for preventive

interventions. Ever since menopause has been recognized as a CV risk factor in women, there have been attempts at treating this condition. One important caution to be remembered is that hormone treatment cannot be intended to reverse age-related vascular dysfunction, but may only target dysfunction owing to a loss of female sex hormones.[100] The latter, on the other hand, may not benefit to the vascular system under aging conditions. In an attempt to analyze both conditions, I introduced the role played by estrogen in the vascular system in the previous section of this thesis. Next, I will describe key operating mechanisms during vascular aging.

1.4.1 Aging and Systemic Inflammation

"Every day you get older… that's a law"

- Butch Cassidy and the Sundance Kid, movie (1969)

Systemic inflammatory burden increases with age.[101] Although the question "why do we age" is likely as old as humanity, to date none of the proposed theories can fully explain all aspects of the complex aging process. Among the dominating views is the theory of immune senescence. Innate and adaptive immunity are the major defence mechanisms against endogenous and environmental threats. Interestingly, during aging, adaptive immunity significantly declines (a process called immunosenescence), whereas innate immunity appears to be activated inducing a pro-inflammatory phenotype (termed inflamm-aging).[102] Thus, aging is associated with a complex and paradoxical remodelling

in the immune system, where immunodeficiency coexists with chronic inflammation (i.e. immune hyperactivation). A resulting decrease in immune competence underlies the pathogenesis of many age-related chronic diseases, including CVD. From this theory, however, it appears that immune/inflammatory changes may be secondary manifestations of another aging process fundamental to all cells (indeed, complex immune systems are not present in all the organisms sharing aspects of aging). It is thought that free radical generation during aerobic metabolic reactions causes direct oxidative damage to DNA, proteins, and lipids. Over time, the damage accumulates resulting in altered function of enzymes and other essential biomolecules, cell membranes, induction of inflammatory defence responses, and cellular senescence (i.e. cessation of proliferation).[103] Cumulative molecular and cellular alterations manifest at the systemic level as decline in organ function, predisposition to degenerative diseases, etc. This theory finds support, in part, from experimentation with calorie-restricted animals, which exhibit a delay in the development of age-related changes, largely owing to reduced metabolic rate, oxygen consumption, and inflammation.[104] A link between the oxidative stress theory and the molecular inflammatory theory of aging may also lay in the activation of redox-sensitive transcriptional factors (e.g., NF-kB) by the excessive levels of ROS in aging, which cause up-regulation of pro-inflammatory genes and massive generation of their products.[105] In turn, pro-inflammatory factors may activate a positive feedback loop to further augment ROS generation and tissue damage.

Chronic inflammation in aging is typically assessed by measuring circulating and tissue levels of inflammatory biomarkers. One of the primary markers of vascular inflammation is infiltration of the vessel intima with leukocytes and activated macrophages, as well as elevated cytokines (e.g., TNF, IL-1β) and acute phase proteins (e.g., CRP, IL-6). To note, chronic systemic inflammation in aging is associated with substantially lower concentrations of these biomarkers than would be generated during an acute condition. Nonetheless, this increased baseline inflammatory activity in aging may be readily amplified by acute stimuli. To date, although positive correlations between inflammatory status and age-related CVD have been well established, the linking molecular pathways are not fully delineated.

1.4.1.1 Vascular remodelling. Pro-inflammatory, pro-oxidant and hemodynamic forces throughout a lifetime trigger vascular wall remodelling. The latter is a multifactorial process, influenced by (in addition to aging) other known CV risk factors. The critical steps in the long-term vessel remodelling include early recruitment of blood monocytes into the intima, their ingestion of lipids to form lipid-rich macrophages (a.k.a. foam cells), release of inflammatory mediators that promote recruitment of VSMC and multiple types of immune cells into the intima, and cell proliferation. Stimulated vessel wall cells themselves become a pathological source of inflammatory and growth-promoting factors. Vascular remodelling in different vascular beds, that possess distinct functions and compensatory capacities, may engage different mechanisms. For example, large arteries, exposed to high pressures and oscillatory flow, are prone to

adaptive hypertrophic remodelling, where excessive extracellular matrix (ECM) augments lipid entrapment, retention and oxidation in the intima. Disturbed shear stress at artery branching sites is another stimulus undermining the protective functions of the endothelium and contributing to atherosclerotic remodelling. Thus, structural changes in the aging conduit vasculature include increased wall thickness (intima/media ratio) due to extensive cell migration, proliferation and ECM deposition. Furthermore, impaired ECM metabolism leads to ineffective matrix proteins structure characterized by collagen cross-linking, elastin fractures and calcium deposition. Indeed, carotid intima/media thickness and calcification scores are useful non-invasive markers to monitor the extent of vascular remodelling and associated CV risk in experimental and clinical settings. One functional implication of this remodelling is altered passive vessel mechanics, including reduced distensibility and stiffening, which impacts blood pressure. In contrast, small resistance arteries that are prone to vasoconstriction (due to myogenic, neurohumoral stimuli, etc.) undergo eutrophic or inward remodelling, where constricted vessel becomes permanently embedded in the remodelled ECM. The main functional implication of this remodelling is an increased peripheral resistance, which contributes to a vicious cycle of hypertension.

In either type of vascular remodelling in aging (typically both are present to varying degrees), the proteolytic enzymes MMPs play leading roles. Their inducibility in an inflammatory milieu, ability to degrade ECM components and participate in important signal transduction pathways, both chronic and acute, will be detailed later in the thesis (section 1.5). Interestingly, estrogen replacement in

Ovx or aging rats showed attenuation of remodelling processes in both conduit and resistance vascular beds, as measured by improved distensibility in isolated vessel preparations.[106, 107] In agreement, a sub-study of the WHI trial demonstrated decreased coronary artery calcification in early postmenopausal women (50-59 years old) who received 7-year estrogen replacement (versus placebo).[108] Estrogen was thought to regulate ECM composition via modulation of MMP activity in the vessel wall,[109] however the mechanisms are not known.

Along with structural remodelling, aging also has an impact on functional vasoactive mechanisms, as the vascular phenotype is dynamic and is constantly being adjusted to environmental factors.

1.4.1.2 Endothelial dysfunction. Vascular endothelial function deteriorates with aging. Endothelial cells regulate inflammatory responses, as they form a critical interface between the blood with circulating immune factors and vascular tissues. In addition, the endothelium has many synthetic and metabolic functions, and closely interacts with adjacent VSMC and the ECM, modulating their functions. Endothelial activation by age-related stimuli (e.g., cytokines, ROS, hemodynamic factors, etc.) may impair many endothelium-mediated vasoprotective functions that precede the clinical onset of CVD. Studies in humans and animals have demonstrated a progressive decline in endothelial NO-mediated vasodilatation in both conduit and resistance arteries with age. Interestingly, experiments in older animals and humans have shown that antioxidant or anti-inflammatory therapy can improve endothelium-dependent relaxation, supporting a link between inflammation and vascular dysfunction in

aging.[68] Moreover, reduced endothelium-mediated responses are evident independent of the vessel structural changes or the VSMC function, as relaxation elicited via endothelium-independent pathways (e.g., sodium nitroprusside) seems to be less affected by aging in some studies.[100] Overall, impaired endothelial vasodilator pathways (including NO), along with the increased generation of vasoconstrictor products (including ET) in the aging endothelial cells contribute to a pro-constrictor vascular phenotype.

In summary, aging is a complex process characterized by sustained pro-inflammatory and pro-constrictor changes in the vascular microenvironment. The following section will describe additional details of the synthesis and significance of NO and ET in vascular dysfunction.

1.4.2 Vasoactive Mediators in Aging

ET and NO are natural, interdependent counterparts in vascular function. An imbalance between these two mediators is a characteristic of endothelial dysfunction, and has been implicated in the progression of CVD.

1.4.2.1 Endothelin-1 generation.[*] ET exerts potent vasopressor, pro-inflammatory and pro-oxidant effects on the vascular system mediated through its specific receptors. The structure, production and vasoconstrictor role of ET were described in the seminal publication by Yanagisawa et al. in 1988 in Nature,[110] and are illustrated (with updates) in Figure 1-3. In humans, of three different

[*] *Modified from the review: O Lekontseva, S Chakrabarti, ST Davidge. AJP Regul Integr Comp Physiol 2010;298(3):R509-16.*

proteins in the ET family (ET-1, ET-2 and ET-3), ET-1 has been established as the principal isoform in the vascular system.[111] Classically, it is generated in the endothelial cells via a number of intermediates with varying (typically low) bioactivities. PreproET-1 (ppET-1) gene transcription yields mRNA encoding for a 212-amino acid peptide, which after removal of its signal sequence forms proET-1, which is further cleaved to big ET-1 (bET-1, a 38-amino acid molecule). The latter can be found in systemic circulation at low levels, but lacks significant bioactivity. Further proteolytic removal of C-terminal residues, classically by ET-converting enzymes (ECEs), results in formation of the mature $ET\text{-}1_{[1\text{-}21]}$.[112, 113] Alternative cleavage of bET-1 by MMP or chymase leads to production of extended-length isoforms, $ET\text{-}1_{[1\text{-}32]}$ and $ET\text{-}1_{[1\text{-}31]}$.[114, 115] Although endothelial cells are considered the primary physiological source of vascular ET-1, VSMC, fibroblasts, and inflammatory cells are capable of ET-1 production under pathological conditions.[116-118] Effects of ET-1 are mediated by the two G protein-coupled receptors, ET_AR and ET_BR.[119] In the vasculature, ET_AR are typically expressed by VSMC, whereas ET_BR are located on both VSMC and endothelial cells. The vasopressor, mitogenic and inflammatory activity of ET-1 has principally been associated with the VSMC ET_AR (and to a lesser extent, VSMC ET_BR). In contrast, endothelial ET_BR mediate release of endothelial vasodilators (such as NO) contributing to vascular relaxation. In normal conditions, the balance between ET_BR-dependent pro-constrictor and pro-dilator pathways is thought to be predominated by its endothelial signaling.[120] In addition, endothelial ET_BR mediate anti-proliferative pathways and ET-1 clearance.[121, 122] However,

this vasoprotective mechanism may be impaired in endothelial dysfunction associated with aging.

The ET-1 system plays physiological role in the maintenance of vascular tone. Given its potent and long-lasting pro-constrictor actions, baseline ET-1 synthesis is low. Aging is associated with augmented ET-1 activity, as evident from higher endogenous ET-1 levels (mRNA and protein) in both plasma and vascular tissue in males and females (humans and rodents), together with an altered ET_AR to ET_BR ratio.[123-127] A recently described C-terminal proET-1 fragment (CT-proET-1), a stable side cleavage product that has been proposed as a better marker of ET-1 synthesis,[128, 129] correlates with poorer clinical prognosis in aging individuals with CVD.[130-132] Interestingly, increased ET-1-dependent vascular tone has been found even in clinically healthy older men.[133] The mechanisms stimulating the ET-1 system in aging are not fully understood. In activated endothelial cells, increased ET-1 can result from an upregulated gene transcription, rapid release of the preformed intracellular stores[134] and/or by means of posttranslational proteolytic maturation. For instance, ppET-1 can be markedly induced by various age-related mechanical and chemical stimuli to the endothelial cells, including disturbed shear stress, cytokines and ROS.[135-137] In addition, increased expression/activity of ECE and non-ECE proteases (e.g., MMP-2 and -9) capable of bET-1 cleavage into bioactive ET-1 has been detected in the aging vasculature.[138-140] However, the functional implications of MMP-dependent ET-1 production in aging have not been demonstrated, and are of particular interest to my research.

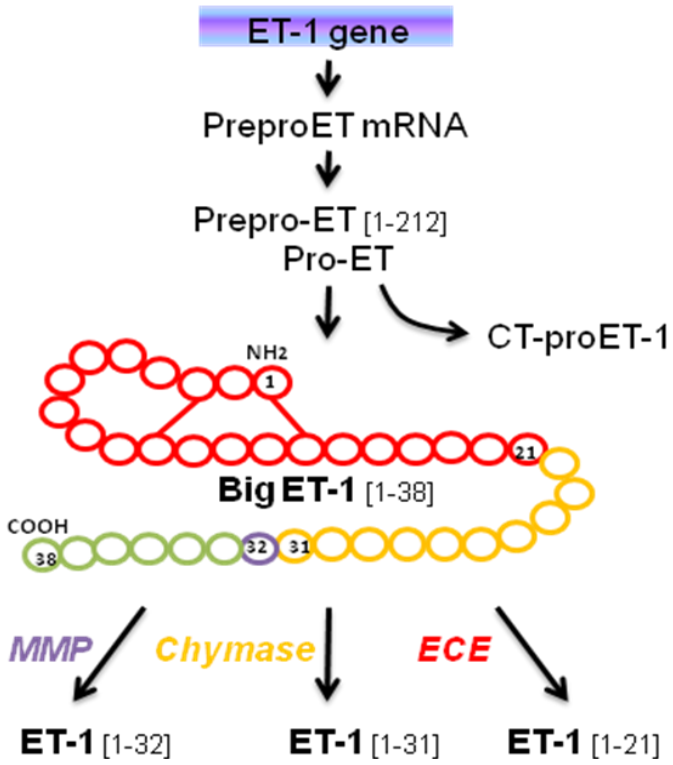

Figure 1-3. Stepwise generation of ET-1

ET-1 is classically a 21-amino acid peptide with free amino- and carboxy-termini and 2 disulfide bonds formed between Cys residues. It is the product of a gene on chromosome 6 that encodes preproET-1, which yields proET-1 after removal of its signal sequence, and is further cleaved by a dibasic pair-specific endopeptidase to form big ET-1. ET-1 is generated from big ET-1 by ECEs and is excreted predominantly at the basolateral surface of endothelial cells. Alternative enzymatic pathways mediated by vascular chymase and MMP process big ET-1 to bioactive 31- and 32-amino acid ET-1. Although not fully shown here, processing of proET-1 can also be more complex, giving rise to other fragments besides big ET-1.[128] For example, C-terminal proET-1 (CT-proET-1) is proposed as a prognostic marker in CVD to assess ET-1 levels.[132]

Important to consider, ET-1 is a complex system tightly linked to other vascular mediators, most notably NO. The constitutive synthesis of NO by endothelial cells tonically inhibits ET-1 activity at various levels from ET-1 gene expression to receptor binding and downstream signaling as recently reviewed by S Bourque and colleagues.[141] In conditions of diminished bioavailable NO, the deleterious potential of ET-1 is unmitigated and contributes to vascular dysfunction via multiple mechanisms. This will be discussed next, following a brief summary of NO biosynthesis, including its sources in the vasculature.

1.4.2.2 Nitric oxide generation. Independent discoveries by Furchgott, Ignarro and Moncada in the 1980's, led to the identification of NO, a short-lived lipophilic gaseous mediator playing an important vasodilatory and anti-inflammatory role in the vascular system.[142-144] NO is released from the endothelial cells both under resting conditions as well as in response to stimulation with a variety of agonists (e.g., acetylcholine, bradykinin) or shear stress. It then acts on vascular cells in an autocrine and paracrine manner. NO induces cGMP-mediated relaxation of VSMC acting primarily via its intracellular receptor, soluble guanylyl cyclase (sGC). Extensive interactions of NO with other vasoactive factors and neural inputs determine the overall vessel tone and blood flow. Besides sGC, NO signals in multiple other ways such as binding to other haem-containing enzymes (e.g., mitochondrial cytochrome c oxidase), chemical modification of proteins via S-nitrosylation, and generation of reactive oxygen/nitrogen species.[145] NOS is the key enzyme catalazing NO production, of which three genetically distinct isoforms have been identified and cloned:

neuronal (nNOS), inducible (iNOS), and endothelial (eNOS). These NOS isoforms were first demonstrated in the brain, macrophages and endothelium (respectively), but have later been found widely distributed in other cell types.[146] The eNOS and nNOS are constitutive enzymes present in the vascular tissue, whereas expression of iNOS depends on the *de novo* synthesis largely induced upon pathological, inflammatory stimulation. A more comprehensive comparative description of NOS enzymes is provided in a separate section (1.6). It is important to note, although eNOS has been considered the main source of vascular NO, nNOS is emerging as another, previously unrecognized intrinsic vascular source. To date, little is understood about the specific nNOS contribution to vascular function in health and disease. However, given certain similarities as well as substantial differences between eNOS and nNOS, it is likely that these enzymes may serve distinct vascular roles, a theme which is further explored in this thesis.

As stated earlier in the introduction, impaired NO bioavailability is the cornerstone of endothelial dysfunction associated with aging and CVD. Many mechanisms can interfere with NO production or accelerate NO consumption in the aging vascular system, thus resulting in decreased net bioavailable NO. To name a few, the mechanisms range from the limited substrate (L-arginine) for NO biosynthesis, altered expression or dysfunction/uncoupling of NOS (included in section 1.6) and rapid scavenging of NO by free radicals. One important consequence of the loss of endothelial NO with aging, beyond reduced NO-mediated vasorelaxation, is reduced inhibition of the ET-1 system. Indeed, the

reciprocal relationship between the two vasoactive mediators has been long noted, and some of the unfavorable outcomes of the altered balance between ET-1 and NO in aging are discussed below.

1.4.2.3 Endothelin-1 and nitric oxide in vascular dysfunction.[*] Increased bioactive ET-1 contributes to vascular dysfunction via multiple pathways (Figure 1-4). Beyond its direct hemodynamic effects, ET-1 is involved in vascular oxidative stress and inflammatory activity, oxidized low density lipoprotein (oxLDL) uptake, mitogenic stimulation of VSMC and fibrotic processes. Antagonistic interactions between ET-1 and NO were shown at many levels. Although ET-1 can mediate a transient release of NO via endothelial ET_BR, in the long term ET-1 reduces eNOS expression and NO production in endothelial cells.[147, 148] A study in healthy volunteers found impaired endothelium-dependent relaxation following 30-min intrabrachial infusion of ET-1, which was prevented by co-administration of an anti-oxidant.[149] ET-1 can generate ROS through activation of NADPH oxidase; and in turn, ROS stimulate ET-1 production, forming a vicious cycle.[150] Indeed, among the many deleterious effects of oxidative stress is rapid consumption of NO by the superoxide radical yielding peroxynitrite, a powerful inflammatory molecule. ET-1 has been further implicated in vascular inflammation via direct activation of macrophages and induction of pro-inflammatory mediators.[151] Cytokines provide a positive feedback to further stimulate ET-1 system, components of which were found abundantly expressed

[*] *Modified from the review: O Lekontseva, S Chakrabarti, ST Davidge. AJP Regul Integr Comp Physiol 2010;298(3):R509-16.*

in areas undergoing active atherosclerotic remodelling.[152, 153] Similar bi-directional interactions have been described with other ET-1 targets. For example, ET-1 augments endothelial uptake of oxLDL, which stimulate ET-1 production.[154, 155] Moreover, dual ETR antagonism improves endothelial function in hyperlipidemic animals,[156] whereas lipid-lowering statin therapy reduces ppET-1 mRNA in endothelial cells[157] and ET-1-dependent vasoconstriction in aorta.[158] Another example of reciprocity is ET_AR-mediated MMP activation, which contributes to pro-fibrotic effects of ET-1 on the vascular ECM.[159] On the other hand, MMPs are known to mediate production of bioactive ET-1 from precursors, which may dominate in certain vascular conditions.[160]

In summary, a vicious cycle of oxidative stress, inflammation, NO deficiency and ET-1 activation is central to the pathogenesis of vascular dysfunction in aging. Given the rate-limiting role of posttranslational ET-1 processing, it is likely that the availability of proteolytic enzymes is an important regulator of ET-1-mediated vascular events. Essential for this thesis, the discovery of the bET-1 cleavage pathway via MMP raises the possibility of its greater weight in the aging vasculature, which is known to undergo extensive MMP-dependent ECM reorganization. A synopsis on the role of MMPs in vascular regulation will follow. Particularly relevant for the generation of my hypothesis, MMP activity is inducible upon inflammatory stimulation that characterizes aging phenotype. Altogether, it will frame my proposal that MMPs contribute to vascular dysfunction in aging via ET-1, a pathway that is potentially impacted by estrogen due to its anti-inflammatory effects. On the other hand,

nNOS function may be important for vascular NO bioavailability. Although limited, evidence will be reviewed, supporting the hypothesized role for nNOS in vascular dysfunction associated with aging and estrogen deficiency. Thus, identifying new roles for old vascular enzymes (MMP and nNOS) in women's CV health, which is greatly influenced by the hormonal status and the aging factor, is the main focus of this thesis research.

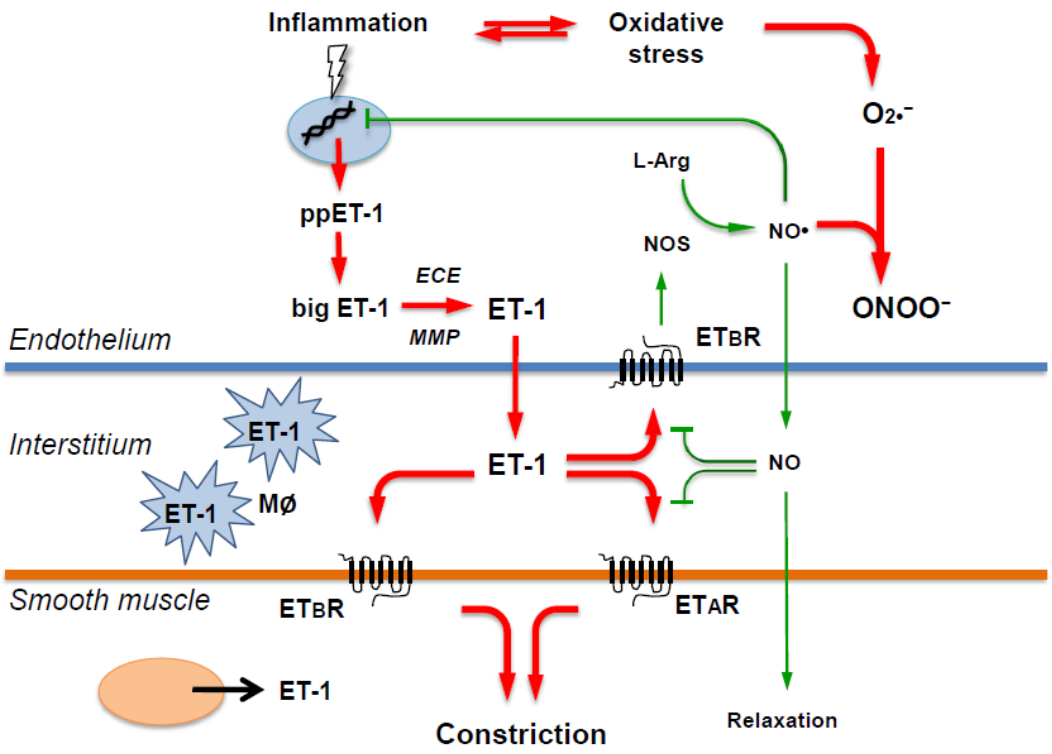

Figure 1-4. ET-1, NO and vascular dysfunction in aging

ET-1 and NO exert physiologically antagonistic effects in the vasculature interacting on several levels. There is increased ET-1 with reduced NO production in dysfunctional endothelial cells in aging. VSMC and macrophages (MØ) become additional sources of ET-1. Beside the ECE-mediated ET-1 generation, MMP may play an increased role in big ET-1 processing, as these enzymes are activated in the aging vasculature. Smooth muscle ET_AR and the ET_BR mediate greater vasoconstriction, as well as contribute to vascular oxidative stress. Increased formation of superoxide ($O_2•^-$) further undermines NO bioavailability by forming peroxynitrite ($ONOO^-$). Collectively, the balance of ET-1 effects is shifted towards more vasoconstriction, inflammation and oxidative stress in the vicious cycle of endothelial dysfunction.

1.5 MATRIX METALLOPROTEINASES IN VASCULAR REGULATION

1.5.1 MMP Family: an Overview

MMPs are a family of zinc-containing endopeptidases that degrade ECM proteins and many other substrates (likely more than are currently known) with diverse physiological and pathological roles. Its first member was discovered in 1962 in amphibian tissues by Gross and Lapiere, who reported collagen-degrading activity in the culture medium of a tadpole undergoing morphogenesis.[161] The identified activity is now known as interstitial collagenase (MMP-1), the enzyme with a unique ability to proteolyse the collagen triple helix at neutral pH. To-date, the MMP family has grown to include 28 members, MMP-1 through -28, which share structural similarities (Figure 1-5), but differ in their expression profile and substrate preferences. Although originally sub-classified based on their ECM proteolytic specificities (e.g., collagenases, gelatinases, stromelysins, matrilysins, and the membrane-type (MT-) MMPs), MMPs have been discovered to cleave a growing number of non-matrix targets, including both extra- and intracellular proteins. For example, processing of cell surface molecules such as growth factors and cytokines may release their bioactive fragments and modulate important signaling pathways. As understanding of these non-classical (non-ECM) substrates and the consequences of their cleavage increases, it becomes clear that MMP activities are not limited to long-term matrix remodelling (such as vascular remodelling in aging). Beyond that, MMPs regulate rapid processes, such as vascular tone, via posttranslational

proteolytic modification of vasoactive peptides (including bET-1). Thus, a detailed understanding of the biology of MMPs in the vascular system, including their regulation and substrate selectivity, is important and may lead to translational outcomes.

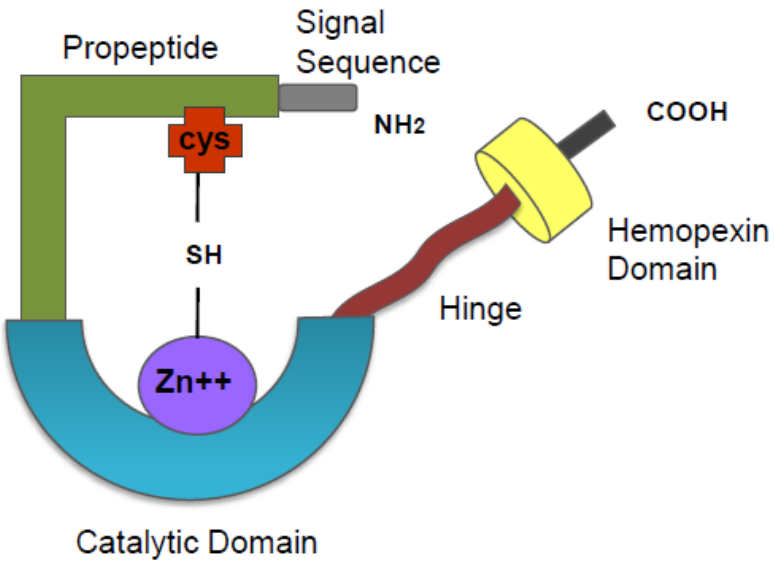

Figure 1-5. Schematic structure of MMP (latent state)

The N-terminal domain typically contains a signaling sequence, which allows for extracellular transport of the enzyme. All MMPs are produced in a zymogen form with a highly conserved propeptide domain, which contains a cysteine switch. The catalytic domain of all MMPs contains Zn^{2+} associated with the Cys residue. Disruption of this bond by proteolytic removal of the propeptide or oxidation of the thiol groups exposes Zn^{2+} for potential interactions, thereby activating the pro-enzyme. Additionally, the catalytic domain of the gelatinases (MMP-2 and -9) contains three fibronectin repeats facilitating collagen binding and cleavage. Except for the matrilysins (MMP-7 and -26), MMPs contain a flexible hinge region with hemopexin domain linked to a C-terminal tail. MT-MMPs additionally possess transmembrane and cytosolic domains at their C-terminus.

1.5.2 Mechanisms of MMP Regulation

1.5.2.1 Expression and gene regulation. MMPs are ubiquitous enzymes, expressed in many cells and tissues, including vascular and blood cells. Of this diverse family, MMP-2 and MMP-9 (a.k.a. gelatinases A and B) have been identified as important players in CVD, where chronic remodelling occurs as a part of the inflammatory process. In the vasculature, MMP-2 is constitutively present in endothelial cells, VSMC, and fibroblasts. MMP-9 is normally found in leukocyte granules, however can be induced in endothelial and other vascular cells upon inflammatory stimulation. In addition, there is evidence of several other MMPs in the vascular tissue, such as MMP-1 (interstitial collagenase), MMP-3 (stromelysin-1), MMP-7 (matrilysin-1), and MMP-8 (neutrophil collagenase), which collectively enables metabolism of all the components of the ECM. However, most MMPs have low expression in quiescent tissues.

Indeed, a common feature of the MMP enzymes is their gene inducibility. The promoter regions of many MMP genes contain binding sites for transcription factors such as NF-κB and AP-1, which play important roles in the regulation of MMP expression. The major activators for these transcriptional factors and MMP synthesis in vascular cells include pro-inflammatory cytokines, ROS, growth factors, mechanical stress (e.g., blood pressure) and chemical stimuli (e.g., hypoxia, hyperglycemia, oxLDL). Interestingly, although ROS generally cause an increase in AP-1 levels and nuclear translocation of NF-κB, oxidative stress can also reduce the transcriptional activity of these factors (thus downregulating MMP) through direct oxidation of Cys residues within their DNA-binding

domain.[162] Thus, regulation of MMP expression appears to involve complex and not yet fully understood mechanisms.

1.5.2.2 Posttranslational activation and inhibition. Given their potentially destructive properties as proteases, MMPs are secreted as inactive or zymogen forms (proMMPs). The pro-form of MMP carries a propeptide domain (with a cysteine switch) that shields the neighbouring catalytic domain (containing Zn^{2+}), thus preventing its access to substrate (Figure 1-5). Removal of the propeptide (by other MMPs or proteases) in the pericellular or extracellular compartment frees the catalytic Zn^{2+} for interactions, thereby activating the enzyme. This proteolytic mechanism of activation (so-called cysteine switch) is believed to be common to all MMPs, and yields truncated enzymatically active forms.

Besides proteolytic processing, oxidative/nitrosative stress can activate proMMPs by reacting with the critical cysteine groups in the propeptide disrupting their binding to the catalytic Zn^{2+}.[163] This posttranslational modification may result in full-length active MMPs, and has been demonstrated for, at least, MMP-1, -2, -8, and -9 (all expressed in the human vasculature). Interestingly, it has been found that lower levels (1-10 µmol/l) of peroxynitrite activate full-length MMP-2 by S-glutathiolation of Cys, whereas higher concentrations (>100 µmol/l) inactivate it, possibly via the nitration of Tyr residues.[164] It is thought that oxidative activation of MMP can occur within the cell, which raises the possibility of intracellular roles/targets for MMPs, a few of them recently described.[165] Moreover, oxidative stress can interfere with MMP activity by causing alterations in the endogenous tissue inhibitors of MMPs (TIMPs).[166]

Another posttranslational modification modulating MMP activity is phosphorylation, where several kinases, including PKA and PKC, have been implicated.[167, 168] However, the role of MMP phosphorylation in the vasculature remains to be understood.

Activity of MMPs *in vivo* can be modulated by their physiological inhibitors, TIMPs. The four identified members of the TIMP family (TIMP-1 through -4) are small (20-30 kDa) proteins; all are present in the vascular system and inhibit MMPs by forming 1:1 molecular binding complexes. Although there is some binding preference of TIMP-2 to MMP-2, and TIMP-1 to MMP-9, generally TIMPs do not demonstrate specificity for any particular MMP. Interestingly, at lower concentrations, TIMP may participate in activation of MMP, as exemplified by the cell surface activation of a 72 kDa proMMP-2 via formation of a complex with TIMP-2 and MT1-MMP. A second molecule of MT1-MMP then interacts with this complex and cleaves the propeptide from MMP-2 resulting in its 64 kDa active form. The binding of other cell or matrix proteins to the hemopexin domain of MMP-2 can facilitate the activation process. In addition, other vascular proteases (e.g., heparin, thrombin, factor Xa) play a role in MMP-2 activation. Besides TIMPs, other endogenous MMP inhibitors are known, including α2- macroglobulin (which removes active MMP forms from the circulation) and, possibly, caveolin-1 (which maintains MMP-2 inactive in association with caveolae).

1.5.2.3 Estrogen and MMP. Although still hypothetical, vascular MMP activity may be modulated by estrogen. For example, a recent study found significantly lower MMP-2 (mRNA, protein and gelatinolytic activity) in

conditioned media from female compared to male rat aortic smooth muscle cells stimulated with IL-1β for 48 hrs.[169] Whereas exogenous E2 did not alter MMP-2 activity in male or female IL-1β stimulated VSMC, chronic *in vivo* E2 exposure greatly reduced male aortic MMP-2 activity. From this study, differences in MMP-2 were speculated as a mechanism for sex disparities in abdominal aortic aneurism formation. The mechanisms whereby estrogen influences MMPs activity are not understood, but may potentially be mediated through its effects on inflammatory factors, a hypothesis to be tested in this thesis (Chapter 3).

In summary, the mechanisms regulating MMP activity in the vasculature appear complex. The net proteolytic activity in the tissue is likely determined by the interplay and balance between endogenous MMP activators and inhibitors.

1.5.3 MMP-2: Vascular Targets and Effects

1.5.3.1 Vascular tissue remodelling. As briefly introduced in the section 1.4.1.1, the ECM is important as a structural support for vascular tissues, and serves many signaling functions regulating cell behaviour. ECM integrity is maintained by a dynamic equilibrium between matrix protein synthesis and degradation. Changes in physiological conditions (including those of aging) modulate MMP activities, causing selective processing of ECM, release of growth factor depots and directed cell migration. What normally is a well coordinated process with precise spatial and temporal MMP activation and inactivation may otherwise (i.e. dysregulated MMPs) become a maladaptive remodelling. Beside matrix metabolism, MMP activity may guide vascular remodelling by modulating

local inflammation. For example, both gelatinases regulate expression of leukocyte adhesion molecules and their interaction with endothelium.[170] Moreover, MMPs cleave and solubilize a range of cytokines and chemokines with either anti- or pro-inflammatory activities. For example, shedding of the membrane-bound form of TNF by gelatinases generates its active form, potentiating inflammation. In contrast, MMP-2-mediated cleavage of the pro-inflammatory factor, monocyte chemoattractant protein-3, results in its truncated form, which acts as a chemokine receptor antagonist.[171] Thus, MMPs act in a long term scale as regulators of chronic vessel wall remodelling.

1.5.3.2 Vascular reactivity. As exemplified above, MMP activity can lead to either gain or loss of function of bioactive molecules through the generation of peptide fragments with modified properties. Interestingly, a number of vasoactive factors can be processed by MMPs allowing for the rapid modulation of vascular tone. Thus, specific cleavage of bET-$1_{[1-38]}$ by MMP-2 yields an intermediate length fragment ET-$1_{[1-32]}$, which may possess distinct vasoconstrictor potency from small ET-$1_{[1-21]}$, a product of the classical ECE-mediated enzymatic pathway. MMP-mediated bET-1 vasoactivity has not been thoroughly investigated, and will be experimentally explored in this thesis. Although, some studies find little role for this pathway in physiological conditions,[172] it may stand out in CVD settings where activity/expression of both, the enzyme and its substrate, is altered. In addition, MMP-2 can contribute to vasoconstriction by degrading a vasodilatory neuropeptide, calcitonin gene-related peptide.[173] Another vasodilator, adrenomedullin, can be broken down by MMP-2 into both

vasodilator and vasoconstrictor fragments.[174] Besides its proteolytic action on vasoactive peptides, MMP can modulate vascular tone via other mechanisms. For example, an MMP role has been described for calcium entry mechanisms[175] and certain signal transduction pathways such as transactivation of EGFR downstream of vasoconstrictor agonists.[176, 177] Taken together, studies suggest that MMPs likely have a greater impact on vascular function than is currently understood.

1.5.3.3 Intracellular targets. Another cutting-edge discovery was that of subcellular localization of bioactive MMP-2, which was found in association with cardiomyocyte sarcomeres and implicated in ischemia-reperfusion injury via proteolysis of troponin I.[178] Whereas degradation of contractile and cytoskeletal proteins is known to underlie acute contractile dysfunction of myocardium following an ischemic attack, little is known about the enzyme(s) involved. More recently, other intracellular targets of MMP-2 in cardiac myocytes have been found, including myosin light chain 1 and titin.[179, 180] Overall, localization to discrete subcellular compartments (including, besides the sarcomere, nucleus and caveolae) suggests additional, yet-to-be understood CV roles for MMP.

Altogether, the later discoveries shift our thinking of MMPs as long-term ECM metabolizers to enzymes with much broader biological roles, including acute actions both outside and inside the cell. Complexity increases as the fragments of degraded proteins may also have unique bioactive roles, potentially important in pathological conditions. This thesis will further investigate whether increased MMP activity, via the ET-1 pathway, is contributing to vascular

dysfunction in aging estrogen deficient animals, using the approach of MMP inhibition in isolated vessels. We then will test whether *in vivo* treatment with estrogen, a potential natural MMP inhibitor, will normalize MMPs and improve vascular function.

The next section will review our second enzyme of interest – NOS, which may contribute to vascular dysfunction in aging from reciprocal to ET, NO-dependent effects.

1.6 NITRIC OXIDE SYNTHASE IN VASCULAR REGULATION

1.6.1 NOS Family: Enzyme Structure and Catalytic Function

NOS, a family of intracellular enzymes catalyzing production of NO, were introduced in the section 1.4.2.2. Three NOS isozymes were rapidly described within 1989-1991, a few years following the breakthrough discovery of NO. The NOS/NO system is a vital player in an unprecedented range of physiological functions including neurotransmission, immune response and defense mechanisms, vascular tone and angiogenesis, to name a few. As a consequence, impaired NO synthesis has been casually implicated in many human diseases including CVD. This section will detail key structural and functional components of NOS that are important to understand pathophysiology.

NOS enzymes are remarkable for the complexity of reactions carried out and the requirement of multiple coenzymes and cofactors.[181] Domain structure of NOS homodimer is illustrated in Figure 1-6. Briefly, each NOS monomer consists of two main regions: reductase and oxygenase domains, as well as linking CaM-binding domain. Although highly homologous, each of the three NOS isoforms has distinctive structural features that reflect their specific *in vivo* functions. Some of the unique characteristics distinguishing eNOS and nNOS that are especially relevant to this thesis will be referred to in the next two consecutive sections (1.6.2 and 1.6.3). The key distinguishing feature between the constitutive and inducible isoforms is dependence on Ca^{2+}. eNOS and nNOS are both activated by an elevation in intracellular Ca^{2+}, followed by binding of Ca^{2+}/CaM. In contrast,

iNOS contains irreversibly bound CaM and is largely independent of Ca^{2+}. iNOS does not appear to play a significant role in the CV regulation under physiological conditions due to its low/absent expression; a conclusion further supported by the lack of an altered phenotype in iNOS-knockout mice.[182] Whereas high levels of iNOS (and NO output) can be induced by inflammatory mediators in most types of vascular cells and have been implicated in acute CV events (such as septic shock), iNOS function is beyond the scope of this thesis, which is aiming to characterize the vascular phenotype undergoing longer term, chronic changes.

The classical reaction catalyzed by NOS is a two-step oxidation of L-arginine to L-citrullin, with the concomitant release of NO. The complex reaction involves the transfer of electrons from NADPH, via the flavins FAD and FMN in the reductase domain, to the haem in the oxygenase domain, where the substrate L-arginine is oxidized.[181] Released NO•, a radical with one unpaired electron, immediately enters its multiple signal transduction pathways. Importantly, NOS can catalyze other side reactions, most notably the generation of $O_2^{\bullet -}$, which has been reported for all NOS isoforms.[183, 184] This is attributed to the dimeric nature of the enzyme, in which the two subunits, as well as isolated reductase and oxygenase domains, are able to function independently.[185] Such reaction (so called uncoupled reaction), where the electron flow is uncoupled from the substrate oxidation, and the conditions favoring it will be discussed using examples of different NOS isoforms in the next section (1.6.2).

In conclusion, NO as a gaseous reactive transmitter is rapidly consumed and not readily stored by the cell, and therefore must be promptly synthesized as

needed. This implies that its biosynthetic enzymes, NOSs, must be the subject of remarkable regulatory control. Although, NOS regulation is not fully understood, evidence indicates that impairment in enzyme function disrupts homeostasis in many vital organ systems. Relevant for this thesis, regulation of eNOS in the vascular system by the female sex steroid estrogen, will be overviewed next.

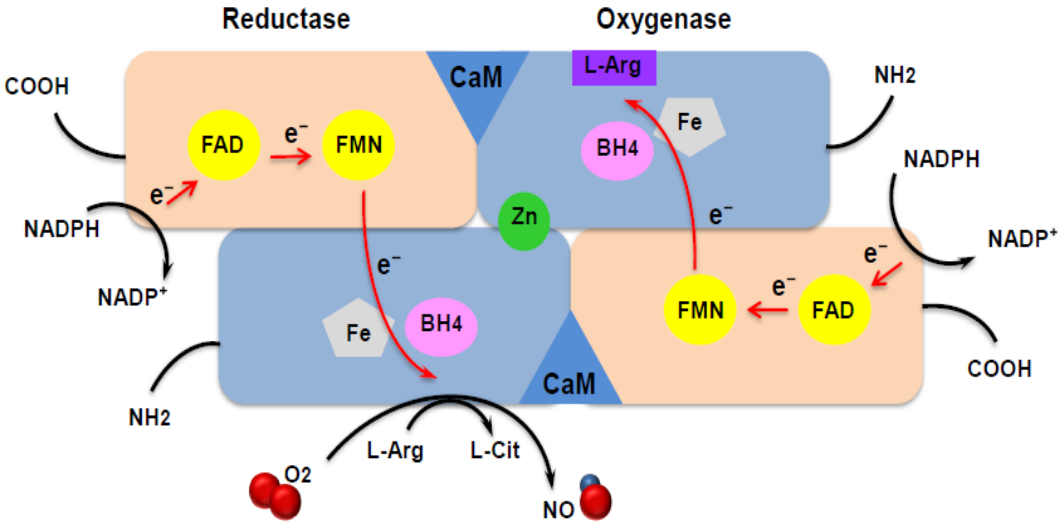

Figure 1-6. Schematic structure of NOS
Adapted from http://www.proteopedia.org/wiki/index.php/Nitric_oxide_synthase

NOS functions as a homodimer. Each of the identical monomers (125-160kDa) has two major domains: a C-terminal reductase domain and an N-terminal oxygenase domain. The reductase domain contains binding sites for NADPH, FAD and FMN that participate in electron transfer to the oxygenase domain of the opposite subunit. The oxygenase domain binds three cofactors: haem, BH_4, and Zn^{2+}, as well as the substrate for oxidation L-arginine. Haem is essential for enzyme dimerization, and Zn^{2+}, BH_4, and L-arginine further contribute to dimer stability. Calmodulin (CaM)-binding domain links the reductase and oxygenase regions, and is important for efficient electron flow. The NO-synthesizing catalytic reaction takes place in the oxygenase domain.

1.6.2 eNOS as a Source of NO in the Vasculature

eNOS is the classic “vasoprotective” NOS isoform expressed in endothelial cells, cardiac myocytes (however, hasn’t been found in VSMC) and blood cells. Despite being a constitutive enzyme, its expression and activity is regulated by multiple biophysical, biochemical and hormonal factors that have been thoroughly studied.

1.6.2.1 eNOS regulation by estrogen. Estrogen stimulates eNOS activity via a number of mechanisms, both genomic and rapid non-genomic, as defined earlier in the introduction. Genomically, via nuclear ERs, estrogen activates eNOS promoter thereby increasing eNOS mRNA synthesis. The eNOS promoter, reportedly, contains an ERE-like motif (but not a complete ERE sequence), as well as binding sites for other transcription factors such as Sp1 that are essential for the promoter activity and appear to mediate ER effects.[186] In contrast, the above study found that other steroid hormones such as testosterone, progesterone or cortisol did not alter eNOS mRNA levels.

On the other hand, estrogen binding to its plasma membrane receptors initiates rapid signaling leading to eNOS activation (Figure 1-7).[187] In its resting state, eNOS is located predominantly at the plasma membrane caveolae, where it closely interacts with caveolin-1, which keeps the enzyme inactive by interfering with CaM binding. Other proteins important for eNOS activity are present in caveolae: acetylcholine/bradykinin receptors, calcium pump, cationic amino acid transporter CAT-1 involved in the up-take of L-arginine, and ERs. In brief, raised intracellular Ca^{2+} via PLC/IP3 pathway downstream of G proteins associated with

the membrane ER (or other agonist receptors) forms a complex with CaM. Moreover, estrogen induces mobilization of heat shock protein (HSP90), an essential chaperone for eNOS. These early Ca^{2+}-dependent events allow dissociation of eNOS from caveolin and translocation into the cytosol, which also conditions the recruitment of activated kinases to phosphorylate eNOS.[188] Subsequent steps in eNOS activation are less Ca^{2+}-dependent.[189] Thus, ER-mediated activation of Akt and MAPK pathways via PI3K and Src signaling causes eNOS phosphorylation at Ser 1177, followed by translocation of the enzyme back to the plasma membrane. Specific posttranslational modifications including myristoylation and palmitoylation (that involve the covalent attachment of fatty acids), distinguish eNOS from the other NOS isoforms, and are necessary for targeting of eNOS to the plasma membrane and fully active enzyme catalyzing oxidation of L-arginine.

1.6.2.2 eNOS uncoupling. At low levels of essential cofactors or the substrate NOS catalyzes uncoupled reduction of oxygen resulting in the release of $O_2^{\bullet -}$. Other mechanisms found to be involved in eNOS uncoupling are: impaired protein-protein interactions with HSP90,[190] dephosphorylation of eNOS at an inhibitory site Thr495 and enzyme redistribution to the cytosolic fraction.[191] The latter two can result from decreased PKC activity in the presence of high oxLDL, which are also known to deplete caveolar cholesterol contributing to displacement of eNOS from the plasma membrane. Interestingly, lipid-lowering therapy with statins has direct protective effects on the endothelium via modulation of caveolin and HSP90 to enhance eNOS-mediated functions.[192]

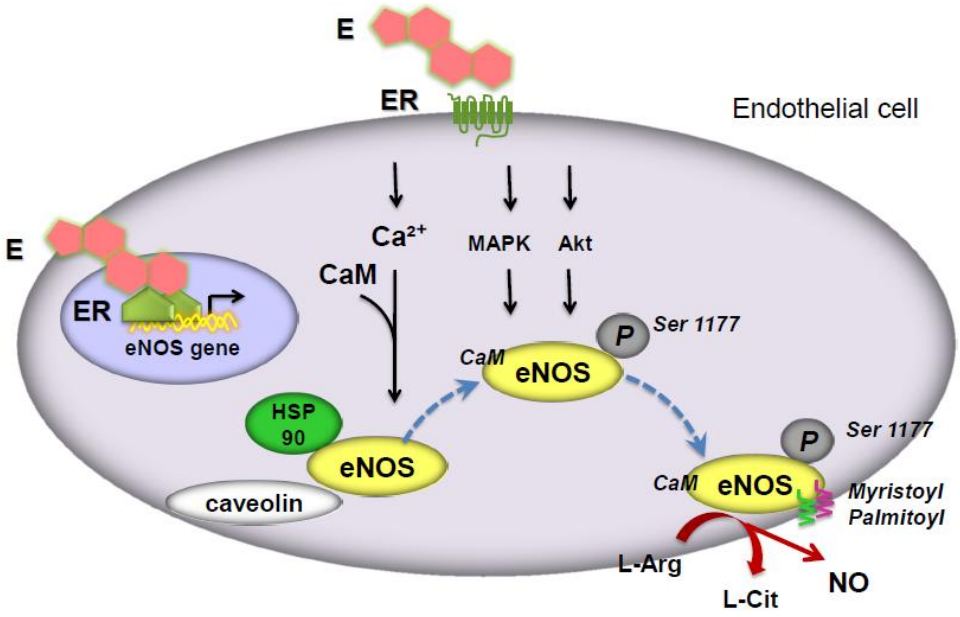

Figure 1-7. Schematic mechanisms of eNOS activation by estrogen

Estrogen (E) activates eNOS via genomic and non-genomic mechanisms. Acting via its nuclear receptors, longer term E exposure up-regulates eNOS gene expression. Acting via plasma membrane receptors coupled to G proteins, E initiates rapid signaling events leading to an increase in intracellular calcium. Calmodulin (CaM) binding to eNOS allows its dissociation from caveolin and HSP90-facilitated translocation to the cytosol, where it undergoes activating site (Ser 1177) phosphorylation mediated via Akt and MAPK pathways. Specific posttranslational modifications such as myristoylation and palmitoylation result in the fully active enzyme at the cell membrane.

Uncoupled reaction is detrimental to vascular function in many ways: superoxide acts as an NO scavenger, as well as a potent pro-oxidant damaging bioactive molecules and generating further reactive oxygen and nitrogen species (ROS and RNS). In a vicious cycle, oxidative stress contributes to NOS uncoupling via mechanisms involving BH_4 oxidation to BH_2, enzyme monomerization, and possible degradation of haem – as shown by Sun et al. on the examples of nNOS and iNOS.[193, 194] The authors also found that various ROS and RNS have differential effects causing NOS uncoupling (i.e. increased $O_2^{\bullet -}$ over NO• production) versus NOS inactivation (i.e. decreased both NO• and $O_2^{\bullet -}$ production). For example, $ONOO^-$ led to potent irreversible NOS inactivation, whereas OH• or H_2O_2 had only mild effects on NOS function, whereas $O_2^{\bullet -}$ induced NOS uncoupling reversible with BH_4 treatment.[193, 194]

Aging is known to induce eNOS uncoupling. As mentioned above, uncoupling has been most consistently understood as a BH_4 or L-arginine deficiency, although it is likely more complex. This is supported by the evidence of improved eNOS function, at least in some experimental settings, with supplementation of the respective factors. Mechanistically, L-arginine deficiency may result from the activity of arginase,[195] which competes with NOS for the substrate: whereas NOS oxidizes L-arginine into L-citrulline and NO, arginase is an enzyme of the urea cycle hydrolysing L-arginine to urea and L-ornithine. The latter re-directs metabolism forming polyamines and L-proline that play a role in VSMC growth and collagen deposition, further contributing to vascular alterations.[196] On the other hand, decreased BH_4 bioavailability in aged

vasculature may result from reduced *de novo* synthesis, loss by oxidation to BH_2, or deficient recycling/regeneration by the enzyme dihydrofolate reductase.[197, 198] These exemplify a few pathways that contribute to eNOS uncoupling in aging.

Interesting to note, there is a difference between NOS isoforms in the rate of the uncoupled reaction. For example, nNOS oxidizes NADPH at a higher rate compared to eNOS or iNOS, explained (in part) by the greater reduction potential of the haem iron in nNOS.[199] The isoform-specific differences in the uncoupled reaction and their pathological relevance are not completely clear, and are addressed experimentally in a chapter this thesis (data included in chapter 5).

Next, I will describe newly emerging evidence on the vascular role of neuronal type NOS, which arguably was overlooked given the stronger research focus on typical “endothelial” NOS in vascular biology for years following the discovery of NOS.

1.6.3 nNOS and Vascular Function

Although the first reports of neuronal-type NOS present in VSMC appeared in the literature in the late 1990's,[200-202] the specific role of vascular nNOS is largely unknown. More recently constitutive nNOS was also shown in endothelial cells.[203, 204] Unlike eNOS, nNOS is predominantly a cytosolic protein, although recently it has been found in the nuclei of certain cell types, including vascular endothelial cells (unpublished lab data). Differential subcellular localization of nNOS may contribute to its diverse functions, although it is a not-yet-understood phenomenon.

Growing evidence suggests that nNOS plays an important role in the local regulation of vascular tone, independent of nNOS-derived NO in the central and peripheral nervous systems. The latter, indeed, is a well described role of nNOS in mediating non-adrenergic non-cholinergic, so-called "nitrergic" relaxation in all types of muscles, including VSMC.[205, 206] Subsequently, a number of studies have shown that nNOS-mediated NO synthesis directly in VSMC modulates their contractility. More recently, endothelial nNOS-derived H_2O_2 has been implicated in vascular tone regulation as an important endothelium-dependent relaxing factor.[207, 208] Differently from eNOS, nNOS is able to form H_2O_2 under physiological conditions in the steps preceding L-arginine oxidation to NO, where H_2O_2 can be formed through self-dismutation of superoxide or result directly from the electron transport.[209-211] Moreover, the first human studies with local intrabrachial or intracoronary infusion of a selective nNOS inhibitor (S-methyl-L-thiocitrulline, SMTC) revealed reduced basal forearm/coronary blood flow without

affecting acetylcholine/substance P-induced vasodilation. On the other hand, a non-selective NOS inhibition affected both basal and agonist-stimulated vessel tone, suggesting that nNOS and eNOS may have distinct *in vivo* roles in regulating basal versus stimulated vascular tone, respectively.[212, 213]

1.6.3.1 nNOS regulation. nNOS is the largest of the three isoforms (160 kDa). The domain structure of nNOS is similar to eNOS (Figure 1-6) with the exception of few unique characteristics. For example, nNOS contains a PDZ motif at the N-terminus, responsible for recognition and association of nNOS with other proteins bearing this domain.[214] This influences the distribution of nNOS to discrete subcellular sites and its enzymatic activity, which is a different mechanism than the fatty acylation-mediated membrane association of eNOS. Similar to eNOS, nNOS can be inhibited by interactions with caveolin, although this has been shown in skeletal muscle.[206] nNOS and eNOS share common mechanisms of activation, such as enzyme dimerization that creates high affinity binding sites for BH_4 and L-arginine and efficient electron flow, Ca^{2+}/CaM binding facilitated by HSP90 association,[215] and phosphorylation at specific sites. Two specific phospho-sites have been found to be important for nNOS activity: 1) inhibitory Ser847 phosphorylation, which is located within the autoinhibitory loop in FMN binding domain and stabilizes the inactive enzyme conformation (potentially similar to inhibitory Thr495 phosphorylation within the CaM site in resting eNOS); 2) stimulatory Ser1417 phosphorylation, which rapidly increases nNOS activity via the mechanism analogous Akt-dependent phosphorylation of eNOS at Ser 1177.

Given many common regulatory mechanisms between eNOS and nNOS, the demonstration of nNOS activation by estrogen in a variety of cell types, particularly neurons[216] and VSMC[49] was not surprising. In a similar manner, estrogen upreguates long term nNOS gene expression via genomic ERE-dependent mechanisms,[217, 218] as well as rapidly modulates phosphorylation of nNOS via kinase pathways downstream of cell surface ERs. However, given the poorly defined role of nNOS in vascular cells, the implications of estrogen-induced nNOS activity are not clear, and will be explored in this thesis. Interesting results were reported in a study investigating the role of nNOS in E2-induced relaxation in endothelium-denuded porcine coronary arteries. White et al. found that inhibition of substrate binding to nNOS (with synthetic L-arginine analogs) did not only attenuate vascular relaxation to E2, but resulted in vasoconstriction, which was reversible with SOD mimetic or superoxide scavenger.[219] These findings suggest that E2 stimulation of uncoupled nNOS further potentiates superoxide generation. It supports the hypothesis that a single molecular mechanism of estrogen action (such as nNOS activation) can lead to opposite physiological outcomes (from good to bad), depending on the vascular conditions.[220] This concept is applied to my studies examining significance of estrogen regulated nNOS activity in a model of vascular aging.

1.7 SUMMARY AND HYPOTHESIS

Both observational and now experimental evidence agree that estrogen actions in the CV system are complex and conditional. Indeed, in addition to its pleiotropic effects on various vascular cells and bioactive molecules, estrogen signaling through a single pathway may lead to different CV outcomes depending on the vascular conditions (e.g., absence or presence of CV risk factors). We hypothesize that aging alters the vascular phenotype – in particular, the function of enzymes involved in the biosynthesis of key vasoactive factors ET-1 and NO, i.e. MMP and nNOS respectively. Therefore, vasoprotection provided by estrogen through MMP- and nNOS-dependent pathways would be impaired in aging.

The specific hypotheses for the studies described in this thesis are:

CHAPTER 2: Combined effects of aging and ovarian deficiency result in a greater MMP-mediated vasoconstriction.

Aim 1: To examine the impact (separate and combined) of aging and ovarian deficiency on vascular responses of small arteries to bET-1.

Aim 2: To examine MMP contribution to the bET-1 reactivity in aging and ovarian deficiency.

Aim 3: To measure gelatinolytic MMP activities in the vascular tissue under the conditions of aging and ovarian deficiency.

CHAPTER 3: MMP-dependent vascular dysfunction in a model of menopause can be improved by E2, potentially via its anti-TNF effects.

Aim 1: To test whether E2 or anti-TNF treatment ameliorates increased vasoconstriction to bET-1 in a model of menopause.

Aim 2: To assess the role of MMP in bET-1 responses in "postmenopausal" animals treated with E2 or TNF inhibitor.

Aim 3: To measure whether E2 or anti-TNF treatments normalize vascular tissue MMP activity.

CHAPTER 4: nNOS mediates estrogen-induced vascular relaxation.

Aim 1: To test whether E2 activates nNOS in isolated endothelial cells.

Aim 2: To validate the presence of nNOS in vessel cross-sections.

Aim 3: To examine role of nNOS in acute vascular relaxation to E2.

Aim 4: To test differences between sexes in nNOS expression and function.

CHAPTER 5: Ovx and E2 treatment have different impacts on young versus aging vascular systems, in part, due to impaired nNOS.

Aim 1: To examine the effect of Ovx with or without E2 treatment in young or aging conditions on acute vascular responses to E2.

Aim 2: To compare role of nNOS in E2 relaxation in young and aging vessels.

Aim 3: To measure vascular tissue nNOS expression and its superoxide-generating capacity in aging and Ovx conditions (i.e. potential uncoupling status).

REFERENCES

1. Pilote L, Dasgupta K, Guru V, Humphries KH, McGrath J, Norris C, Rabi D, Tremblay J, Alamian A, Barnett T, Cox J, Ghali WA, Grace S, Hamet P, Ho T, Kirkland S, Lambert M, Libersan D, O'Loughlin J, Paradis G, Petrovich M, Tagalakis V. A comprehensive view of sex-specific issues related to cardiovascular disease. *Cmaj.* 2007;176(6):S1-44.

2. Knot HJ, Lounsbury KM, Brayden JE, Nelson MT. Gender differences in coronary artery diameter reflect changes in both endothelial Ca2+ and ecNOS activity. *The American journal of physiology.* 1999;276(3 Pt 2):H961-969.

3. Kim ES, Menon V. Status of women in cardiovascular clinical trials. *Arteriosclerosis, thrombosis, and vascular biology.* 2009;29(3):279-283.

4. Zhu BT, Han GZ, Shim JY, Wen Y, Jiang XR. Quantitative structure-activity relationship of various endogenous estrogen metabolites for human estrogen receptor alpha and beta subtypes: Insights into the structural determinants favoring a differential subtype binding. *Endocrinology.* 2006;147(9):4132-4150.

5. Dubey RK, Tofovic SP, Jackson EK. Cardiovascular pharmacology of estradiol metabolites. *The Journal of pharmacology and experimental therapeutics.* 2004;308(2):403-409.

6. Krause DN, Duckles SP, Gonzales RJ. Local oestrogenic/androgenic balance in the cerebral vasculature. *Acta physiologica (Oxford, England).* 2011.

7. Hong S, Didwania A, Olopade O, Ganschow P. The expanding use of third-generation aromatase inhibitors: what the general internist needs to know. *Journal of general internal medicine.* 2009;24 Suppl 2:S383-388.

8. Santmyire BR, Venkat V, Beinder E, Baylis C. Impact of the estrus cycle and reduction in estrogen levels with aromatase inhibition, on renal function and nitric oxide activity in female rats. *Steroids.* 2010;75(12):1011-1015.

9. Yu J, Akishita M, Eto M, Ogawa S, Son BK, Kato S, Ouchi Y, Okabe T. Androgen receptor-dependent activation of endothelial nitric oxide synthase

in vascular endothelial cells: role of phosphatidylinositol 3-kinase/akt pathway. *Endocrinology.* 2010;151(4):1822-1828.

10. Ewer MS, Gluck S. A woman's heart: the impact of adjuvant endocrine therapy on cardiovascular health. *Cancer.* 2009;115(9):1813-1826.

11. O'Lone R, Knorr K, Jaffe IZ, Schaffer ME, Martini PG, Karas RH, Bienkowska J, Mendelsohn ME, Hansen U. Estrogen receptors alpha and beta mediate distinct pathways of vascular gene expression, including genes involved in mitochondrial electron transport and generation of reactive oxygen species. *Molecular endocrinology (Baltimore, Md.* 2007;21(6):1281-1296.

12. Post WS, Goldschmidt-Clermont PJ, Wilhide CC, Heldman AW, Sussman MS, Ouyang P, Milliken EE, Issa JP. Methylation of the estrogen receptor gene is associated with aging and atherosclerosis in the cardiovascular system. *Cardiovascular research.* 1999;43(4):985-991.

13. Kim J, Kim JY, Song KS, Lee YH, Seo JS, Jelinek J, Goldschmidt-Clermont PJ, Issa JP. Epigenetic changes in estrogen receptor beta gene in atherosclerotic cardiovascular tissues and in-vitro vascular senescence. *Biochimica et biophysica acta.* 2007;1772(1):72-80.

14. Ihionkhan CE, Chambliss KL, Gibson LL, Hahner LD, Mendelsohn ME, Shaul PW. Estrogen causes dynamic alterations in endothelial estrogen receptor expression. *Circulation research.* 2002;91(9):814-820.

15. Chakrabarti S, Davidge ST. High glucose-induced oxidative stress alters estrogen effects on ERalpha and ERbeta in human endothelial cells: reversal by AMPK activator. *The Journal of steroid biochemistry and molecular biology.* 2009;117(4-5):99-106.

16. Kato S, Endoh H, Masuhiro Y, Kitamoto T, Uchiyama S, Sasaki H, Masushige S, Gotoh Y, Nishida E, Kawashima H, Metzger D, Chambon P. Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science (New York, N.Y.* 1995;270(5241):1491-1494.

17. Lupien M, Meyer CA, Bailey ST, Eeckhoute J, Cook J, Westerling T, Zhang X, Carroll JS, Rhodes DR, Liu XS, Brown M. Growth factor stimulation induces a distinct ER(alpha) cistrome underlying breast cancer endocrine resistance. *Genes & development.* 2010;24(19):2219-2227.

18. Edwards DP. Regulation of signal transduction pathways by estrogen and progesterone. *Annual review of physiology.* 2005;67:335-376.

19. Shearman AM, Cupples LA, Demissie S, Peter I, Schmid CH, Karas RH, Mendelsohn ME, Housman DE, Levy D. Association between estrogen receptor alpha gene variation and cardiovascular disease. *Jama.* 2003;290(17):2263-2270.

20. Lehtimaki T, Kunnas TA, Mattila KM, Perola M, Penttila A, Koivula T, Karhunen PJ. Coronary artery wall atherosclerosis in relation to the estrogen receptor 1 gene polymorphism: an autopsy study. *Journal of molecular medicine (Berlin, Germany).* 2002;80(3):176-180.

21. Mizukami Y. In vivo functions of GPR30/GPER-1, a membrane receptor for estrogen: from discovery to functions in vivo. *Endocrine journal.* 2010;57(2):101-107.

22. Haas E, Meyer MR, Schurr U, Bhattacharya I, Minotti R, Nguyen HH, Heigl A, Lachat M, Genoni M, Barton M. Differential effects of 17beta-estradiol on function and expression of estrogen receptor alpha, estrogen receptor beta, and GPR30 in arteries and veins of patients with atherosclerosis. *Hypertension.* 2007;49(6):1358-1363.

23. Nilsson S, Makela S, Treuter E, Tujague M, Thomsen J, Andersson G, Enmark E, Pettersson K, Warner M, Gustafsson JA. Mechanisms of estrogen action. *Physiological reviews.* 2001;81(4):1535-1565.

24. Hall JM, McDonnell DP. Coregulators in nuclear estrogen receptor action: from concept to therapeutic targeting. *Molecular interventions.* 2005;5(6):343-357.

25. Dahlman-Wright K, Cavailles V, Fuqua SA, Jordan VC, Katzenellenbogen JA, Korach KS, Maggi A, Muramatsu M, Parker MG, Gustafsson JA. International Union of Pharmacology. LXIV. Estrogen receptors. *Pharmacological reviews.* 2006;58(4):773-781.

26. Heldring N, Pike A, Andersson S, Matthews J, Cheng G, Hartman J, Tujague M, Strom A, Treuter E, Warner M, Gustafsson JA. Estrogen receptors: how do they signal and what are their targets. *Physiological reviews.* 2007;87(3):905-931.

27. Hammes SR, Levin ER. Extranuclear steroid receptors: nature and actions. *Endocrine reviews.* 2007;28(7):726-741.

28. Kim KH, Moriarty K, Bender JR. Vascular cell signaling by membrane estrogen receptors. *Steroids.* 2008;73(9-10):864-869.

29. Chambliss KL, Yuhanna IS, Anderson RG, Mendelsohn ME, Shaul PW. ERbeta has nongenomic action in caveolae. *Molecular endocrinology (Baltimore, Md.* 2002;16(5):938-946.

30. Prossnitz ER, Arterburn JB, Smith HO, Oprea TI, Sklar LA, Hathaway HJ. Estrogen signaling through the transmembrane G protein-coupled receptor GPR30. *Annual review of physiology.* 2008;70:165-190.

31. Fernandez-Patron C. Therapeutic potential of the epidermal growth factor receptor transactivation in hypertension: a convergent signaling pathway of vascular tone, oxidative stress, and hypertrophic growth downstream of vasoactive G-protein-coupled receptors? *Canadian journal of physiology and pharmacology.* 2007;85(1):97-104.

32. Lindsey SH, Cohen JA, Brosnihan KB, Gallagher PE, Chappell MC. Chronic treatment with the G protein-coupled receptor 30 agonist G-1 decreases blood pressure in ovariectomized mRen2.Lewis rats. *Endocrinology.* 2009;150(8):3753-3758.

33. Broughton BR, Miller AA, Sobey CG. Endothelium-dependent relaxation by G protein-coupled receptor 30 agonists in rat carotid arteries. *American journal of physiology.* 2010;298(3):H1055-1061.

34. Lindsey SH, Carver KA, Prossnitz ER, Chappell MC. Vasodilation in Response to the GPR30 Agonist G-1 is Not Different From Estradiol in the mRen2.Lewis Female Rat. *Journal of cardiovascular pharmacology.*57(5):598-603.

35. Haas E, Bhattacharya I, Brailoiu E, Damjanovic M, Brailoiu GC, Gao X, Mueller-Guerre L, Marjon NA, Gut A, Minotti R, Meyer MR, Amann K, Ammann E, Perez-Dominguez A, Genoni M, Clegg DJ, Dun NJ, Resta TC, Prossnitz ER, Barton M. Regulatory role of G protein-coupled estrogen receptor for vascular function and obesity. *Circulation research.* 2009;104(3):288-291.

36. Grodstein F, Manson JE, Colditz GA, Willett WC, Speizer FE, Stampfer MJ. A prospective, observational study of postmenopausal hormone therapy and primary prevention of cardiovascular disease. *Annals of internal medicine.* 2000;133(12):933-941.

37. Faulds MH, Zhao C, Dahlman-Wright K, Gustafsson JA. Regulation of metabolism by estrogen signaling. *The Journal of endocrinology.*

38. Hashimoto M, Akishita M, Eto M, Ishikawa M, Kozaki K, Toba K, Sagara Y, Taketani Y, Orimo H, Ouchi Y. Modulation of endothelium-dependent flow-mediated dilatation of the brachial artery by sex and menstrual cycle. *Circulation.* 1995;92(12):3431-3435.

39. Hayashi T, Fukuto JM, Ignarro LJ, Chaudhuri G. Basal release of nitric oxide from aortic rings is greater in female rabbits than in male rabbits: implications for atherosclerosis. *Proceedings of the National Academy of Sciences of the United States of America.* 1992;89(23):11259-11263.

40. Conrad KP, Joffe GM, Kruszyna H, Kruszyna R, Rochelle LG, Smith RP, Chavez JE, Mosher MD. Identification of increased nitric oxide biosynthesis during pregnancy in rats. *Faseb J.* 1993;7(6):566-571.

41. Kharitonov SA, Logan-Sinclair RB, Busset CM, Shinebourne EA. Peak expiratory nitric oxide differences in men and women: relation to the menstrual cycle. *British heart journal.* 1994;72(3):243-245.

42. Tan E, Gurjar MV, Sharma RV, Bhalla RC. Estrogen receptor-alpha gene transfer into bovine aortic endothelial cells induces eNOS gene expression and inhibits cell migration. *Cardiovascular research.* 1999;43(3):788-797.

43. Chambliss KL, Shaul PW. Estrogen modulation of endothelial nitric oxide synthase. *Endocrine reviews.* 2002;23(5):665-686.

44. Lam KK, Lee YM, Hsiao G, Chen SY, Yen MH. Estrogen therapy replenishes vascular tetrahydrobiopterin and reduces oxidative stress in ovariectomized rats. *Menopause (New York, N.Y.* 2006;13(2):294-302.

45. Monsalve E, Oviedo PJ, Garcia-Perez MA, Tarin JJ, Cano A, Hermenegildo C. Estradiol counteracts oxidized LDL-induced asymmetric dimethylarginine production by cultured human endothelial cells. *Cardiovascular research.* 2007;73(1):66-72.

46. Sobrino A, Oviedo PJ, Novella S, Laguna-Fernandez A, Bueno C, Garcia-Perez MA, Tarin JJ, Cano A, Hermenegildo C. Estradiol selectively stimulates endothelial prostacyclin production through estrogen receptor-{alpha}. *Journal of molecular endocrinology.* 2010;44(4):237-246.

47. Sakuma I, Liu MY, Sato A, Hayashi T, Iguchi A, Kitabatake A, Hattori Y. Endothelium-dependent hyperpolarization and relaxation in mesenteric arteries of middle-aged rats: influence of oestrogen. *British journal of pharmacology.* 2002;135(1):48-54.

48. Nawate S, Fukao M, Sakuma I, Soma T, Nagai K, Takikawa O, Miwa S, Kitabatake A. Reciprocal changes in endothelium-derived hyperpolarizing factor- and nitric oxide-system in the mesenteric artery of adult female rats following ovariectomy. *British journal of pharmacology.* 2005;144(2):178-189.

49. Han G, Ma H, Chintala R, Miyake K, Fulton DJ, Barman SA, White RE. Nongenomic, endothelium-independent effects of estrogen on human coronary smooth muscle are mediated by type I (neuronal) NOS and PI3-kinase-Akt signaling. *American journal of physiology.* 2007;293(1):H314-321.

50. Tsang SY, Yao X, Chan HY, Wong CM, Chen ZY, Au CL, Huang Y. Contribution of K+ channels to relaxation induced by 17beta-estradiol but not by progesterone in isolated rat mesenteric artery rings. *Journal of cardiovascular pharmacology.* 2003;41(1):4-13.

51. White RE, Darkow DJ, Lang JL. Estrogen relaxes coronary arteries by opening BKCa channels through a cGMP-dependent mechanism. *Circulation research.* 1995;77(5):936-942.

52. Han SZ, Karaki H, Ouchi Y, Akishita M, Orimo H. 17 beta-Estradiol inhibits Ca2+ influx and Ca2+ release induced by thromboxane A2 in porcine coronary artery. *Circulation.* 1995;91(10):2619-2626.

53. Yang E, Jeon SB, Baek I, Chen ZA, Jin Z, Kim IK. 17beta-estradiol attenuates vascular contraction through inhibition of RhoA/Rho kinase pathway. *Naunyn-Schmiedeberg's archives of pharmacology.* 2009;380(1):35-44.

54. Kanashiro CA, Khalil RA. Gender-related distinctions in protein kinase C activity in rat vascular smooth muscle. *Am J Physiol Cell Physiol.* 2001;280(1):C34-45.

55. Yung LM, Wong WT, Tian XY, Leung FP, Yung LH, Chen ZY, Yao X, Lau CW, Huang Y. Inhibition of renin-angiotensin system reverses endothelial dysfunction and oxidative stress in estrogen deficient rats. *PloS one.* 2011;6(3):e17437.

56. David FL, Carvalho MH, Cobra AL, Nigro D, Fortes ZB, Reboucas NA, Tostes RC. Ovarian hormones modulate endothelin-1 vascular reactivity and mRNA expression in DOCA-salt hypertensive rats. *Hypertension.* 2001;38(3 Pt 2):692-696.

57. Akishita M, Kozaki K, Eto M, Yoshizumi M, Ishikawa M, Toba K, Orimo H, Ouchi Y. Estrogen attenuates endothelin-1 production by bovine endothelial cells via estrogen receptor. *Biochemical and biophysical research communications.* 1998;251(1):17-21.

58. Wingrove CS, Stevenson JC. 17 beta-Oestradiol inhibits stimulated endothelin release in human vascular endothelial cells. *European journal of endocrinology / European Federation of Endocrine Societies.* 1997;137(2):205-208.

59. Pedersen SH, Nielsen LB, Pedersen NG, Nilas L, Ottesen B. Hormone therapy modulates ET(A) mRNA expression in the aorta of ovariectomised New Zealand White rabbits. *Gynecol Endocrinol.* 2009;25(3):175-182.

60. Vongpatanasin W, Tuncel M, Mansour Y, Arbique D, Victor RG. Transdermal estrogen replacement therapy decreases sympathetic activity in postmenopausal women. *Circulation.* 2001;103(24):2903-2908.

61. Dubey RK, Jackson EK, Gillespie DG, Zacharia LC, Imthurn B. Catecholamines block the antimitogenic effect of estradiol on human coronary artery smooth muscle cells. *The Journal of clinical endocrinology and metabolism.* 2004;89(8):3922-3931.

62. Kaur G, Janik J, Isaacson LG, Callahan P. Estrogen regulation of neurotrophin expression in sympathetic neurons and vascular targets. *Brain research.* 2007;1139:6-14.

63. Strehlow K, Rotter S, Wassmann S, Adam O, Grohe C, Laufs K, Bohm M, Nickenig G. Modulation of antioxidant enzyme expression and function by estrogen. *Circulation research.* 2003;93(2):170-177.

64. Behl C, Skutella T, Lezoualc'h F, Post A, Widmann M, Newton CJ, Holsboer F. Neuroprotection against oxidative stress by estrogens: structure-activity relationship. *Molecular pharmacology.* 1997;51(4):535-541.

65. Chalopin M, Tesse A, Martinez MC, Rognan D, Arnal JF, Andriantsitohaina R. Estrogen receptor alpha as a key target of red wine polyphenols action on the endothelium. *PloS one.*5(1):e8554.

66. Ferreri NR. Estrogen-TNF interactions and vascular inflammation. *American journal of physiology.* 2007;292(6):H2566-2569.

67. Vila E, Salaices M. Cytokines and vascular reactivity in resistance arteries. *American journal of physiology.* 2005;288(3):H1016-1021.

68. Arenas IA, Armstrong SJ, Xu Y, Davidge ST. Chronic tumor necrosis factor-alpha inhibition enhances NO modulation of vascular function in estrogen-deficient rats. *Hypertension.* 2005;46(1):76-81.

69. Kamada M, Irahara M, Maegawa M, Ohmoto Y, Takeji T, Yasui T, Aono T. Postmenopausal changes in serum cytokine levels and hormone replacement therapy. *American journal of obstetrics and gynecology.* 2001;184(3):309-314.

70. Mori M, Tsukahara F, Yoshioka T, Irie K, Ohta H. Suppression by 17beta-estradiol of monocyte adhesion to vascular endothelial cells is mediated by estrogen receptors. *Life sciences.* 2004;75(5):599-609.

71. Xing D, Feng W, Miller AP, Weathington NM, Chen YF, Novak L, Blalock JE, Oparil S. Estrogen modulates TNF-alpha-induced inflammatory responses in rat aortic smooth muscle cells through estrogen receptor-beta activation. *American journal of physiology.* 2007;292(6):H2607-2612.

72. Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, Jackson RD, Beresford SA, Howard BV, Johnson KC, Kotchen JM, Ockene J. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. *Jama.* 2002;288(3):321-333.

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

74. Grady D, Herrington D, Bittner V, Blumenthal R, Davidson M, Hlatky M, Hsia J, Hulley S, Herd A, Khan S, Newby LK, Waters D, Vittinghoff E, Wenger N. Cardiovascular disease outcomes during 6.8 years of hormone

therapy: Heart and Estrogen/progestin Replacement Study follow-up (HERS II). *Jama.* 2002;288(1):49-57.

75. Dubey RK, Imthurn B, Zacharia LC, Jackson EK. Hormone replacement therapy and cardiovascular disease: what went wrong and where do we go from here? *Hypertension.* 2004;44(6):789-795.

76. Dubey RK, Imthurn B, Barton M, Jackson EK. Vascular consequences of menopause and hormone therapy: importance of timing of treatment and type of estrogen. *Cardiovascular research.* 2005;66(2):295-306.

77. Decensi A, Omodei U, Robertson C, Bonanni B, Guerrieri-Gonzaga A, Ramazzotto F, Johansson H, Mora S, Sandri MT, Cazzaniga M, Franchi M, Pecorelli S. Effect of transdermal estradiol and oral conjugated estrogen on C-reactive protein in retinoid-placebo trial in healthy women. *Circulation.* 2002;106(10):1224-1228.

78. Lakoski SG, Herrington DM. Effects of hormone therapy on C-reactive protein and IL-6 in postmenopausal women: a review article. *Climacteric.* 2005;8(4):317-326.

79. Clark JH. A critique of Women's Health Initiative Studies (2002-2006). *Nuclear receptor signaling.* 2006;4:e023.

80. Harman SM, Brinton EA, Cedars M, Lobo R, Manson JE, Merriam GR, Miller VM, Naftolin F, Santoro N. KEEPS: The Kronos Early Estrogen Prevention Study. *Climacteric.* 2005;8(1):3-12.

81. Brinton EA, Hodis HN, Merriam GR, Harman SM, Naftolin F. Can menopausal hormone therapy prevent coronary heart disease? *Trends in endocrinology and metabolism: TEM.* 2008;19(6):206-212.

82. Xu HL, Baughman VL, Pelligrino DA. Estrogen replacement treatment in diabetic ovariectomized female rats potentiates postischemic leukocyte adhesion in cerebral venules. *Stroke; a journal of cerebral circulation.* 2004;35(8):1974-1978.

83. Santizo RA, Xu HL, Galea E, Muyskens S, Baughman VL, Pelligrino DA. Combined endothelial nitric oxide synthase upregulation and caveolin-1 downregulation decrease leukocyte adhesion in pial venules of ovariectomized female rats. *Stroke; a journal of cerebral circulation.* 2002;33(2):613-616.

84. Galea E, Santizo R, Feinstein DL, Adamsom P, Greenwood J, Koenig HM, Pelligrino DA. Estrogen inhibits NF kappa B-dependent inflammation in brain endothelium without interfering with I kappa B degradation. *Neuroreport.* 2002;13(11):1469-1472.

85. Umetani M, Domoto H, Gormley AK, Yuhanna IS, Cummins CL, Javitt NB, Korach KS, Shaul PW, Mangelsdorf DJ. 27-Hydroxycholesterol is an endogenous SERM that inhibits the cardiovascular effects of estrogen. *Nature medicine.* 2007;13(10):1185-1192.

86. Vasquez-Vivar J, Kalyanaraman B, Martasek P, Hogg N, Masters BS, Karoui H, Tordo P, Pritchard KA, Jr. Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. *Proceedings of the National Academy of Sciences of the United States of America.* 1998;95(16):9220-9225.

87. Coma M, Guix FX, Uribesalgo I, Espuna G, Sole M, Andreu D, Munoz FJ. Lack of oestrogen protection in amyloid-mediated endothelial damage due to protein nitrotyrosination. *Brain.* 2005;128(Pt 7):1613-1621.

88. Miller AP, Xing D, Feng W, Fintel M, Chen YF, Oparil S. Aged rats lose vasoprotective and anti-inflammatory actions of estrogen in injured arteries. *Menopause (New York, N.Y.* 2007;14(2):251-260.

89. Sherwood A, Bower JK, McFetridge-Durdle J, Blumenthal JA, Newby LK, Hinderliter AL. Age moderates the short-term effects of transdermal 17beta-estradiol on endothelium-dependent vascular function in postmenopausal women. *Arteriosclerosis, thrombosis, and vascular biology.* 2007;27(8):1782-1787.

90. Pereira IR, Bertolami MC, Faludi AA, Campos MF, Ferderbar S, Lima ES, Aldrighi JM, Abdalla DS. Lipid peroxidation and nitric oxide inactivation in postmenopausal women. *Arquivos brasileiros de cardiologia.* 2003;80(4):406-423.

91. Whittal RM, Benz CC, Scott G, Semyonov J, Burlingame AL, Baldwin MA. Preferential oxidation of zinc finger 2 in estrogen receptor DNA-binding domain prevents dimerization and, hence, DNA binding. *Biochemistry.* 2000;39(29):8406-8417.

92. Garban HJ, Marquez-Garban DC, Pietras RJ, Ignarro LJ. Rapid nitric oxide-mediated S-nitrosylation of estrogen receptor: regulation of estrogen-

dependent gene transcription. *Proceedings of the National Academy of Sciences of the United States of America.* 2005;102(7):2632-2636.

93. Marino M, Ficca R, Ascenzi P, Trentalance A. Nitric oxide inhibits selectively the 17beta-estradiol-induced gene expression without affecting nongenomic events in HeLa cells. *Biochemical and biophysical research communications.* 2001;286(3):529-533.

94. Hong SC, Yoo SW, Cho GJ, Kim T, Hur JY, Park YK, Lee KW, Kim SH. Correlation between estrogens and serum adipocytokines in premenopausal and postmenopausal women. *Menopause (New York, N.Y.* 2007;14(5):835-840.

95. Cid MC, Kleinman HK, Grant DS, Schnaper HW, Fauci AS, Hoffman GS. Estradiol enhances leukocyte binding to tumor necrosis factor (TNF)-stimulated endothelial cells via an increase in TNF-induced adhesion molecules E-selectin, intercellular adhesion molecule type 1, and vascular cell adhesion molecule type 1. *The Journal of clinical investigation.* 1994;93(1):17-25.

96. Traub ML, Santoro N. Reproductive aging and its consequences for general health. *Annals of the New York Academy of Sciences.* 2010;1204:179-187.

97. Giunta S. Exploring the complex relations between inflammation and aging (inflamm-aging): anti-inflamm-aging remodelling of inflamm- aging, from robustness to frailty. *Inflamm Res.* 2008;57(12):558-563.

98. Vural P, Akgul C, Canbaz M. Effects of hormone replacement therapy on plasma pro-inflammatory and anti-inflammatory cytokines and some bone turnover markers in postmenopausal women. *Pharmacol Res.* 2006;54(4):298-302.

99. Prestwood KM, Unson C, Kulldorff M, Cushman M. The effect of different doses of micronized 17beta-estradiol on C-reactive protein, interleukin-6, and lipids in older women. *The journals of gerontology.* 2004;59(8):827-832.

100. Taddei S, Virdis A, Ghiadoni L, Mattei P, Sudano I, Bernini G, Pinto S, Salvetti A. Menopause is associated with endothelial dysfunction in women. *Hypertension.* 1996;28(4):576-582.

101. Anuurad E, Enkhmaa B, Gungor Z, Zhang W, Tracy RP, Pearson TA, Kim K, Berglund L. Age as a Modulator of Inflammatory Cardiovascular Risk Factors. *Arteriosclerosis, thrombosis, and vascular biology.*

102. Boren E, Gershwin ME. Inflamm-aging: autoimmunity, and the immune-risk phenotype. *Autoimmunity reviews.* 2004;3(5):401-406.

103. Harman D. The free radical theory of aging. *Antioxidants & redox signaling.* 2003;5(5):557-561.

104. Ungvari Z, Parrado-Fernandez C, Csiszar A, de Cabo R. Mechanisms underlying caloric restriction and lifespan regulation: implications for vascular aging. *Circulation research.* 2008;102(5):519-528.

105. Chung HY, Sung B, Jung KJ, Zou Y, Yu BP. The molecular inflammatory process in aging. *Antioxidants & redox signaling.* 2006;8(3-4):572-581.

106. Mullick AE, Walsh BA, Reiser KM, Rutledge JC. Chronic estradiol treatment attenuates stiffening, glycoxidation, and permeability in rat carotid arteries. *American journal of physiology.* 2001;281(5):H2204-2210.

107. Zhang Y, Stewart KG, Davidge ST. Estrogen replacement reduces age-associated remodeling in rat mesenteric arteries. *Hypertension.* 2000;36(6):970-974.

108. Manson JE, Allison MA, Rossouw JE, Carr JJ, Langer RD, Hsia J, Kuller LH, Cochrane BB, Hunt JR, Ludlam SE, Pettinger MB, Gass M, Margolis KL, Nathan L, Ockene JK, Prentice RL, Robbins J, Stefanick ML. Estrogen therapy and coronary-artery calcification. *The New England journal of medicine.* 2007;356(25):2591-2602.

109. Lam KK, Cheng PY, Hsiao G, Chen SY, Shen HH, Yen MH, Lee YM. Estrogen deficiency-induced alterations of vascular MMP-2, MT1-MMP, and TIMP-2 in ovariectomized rats. *American journal of hypertension.* 2009;22(1):27-34.

110. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki T. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature.* 1988;332(6163):411-415.

111. Masaki T. Historical review: Endothelin. *Trends in pharmacological sciences.* 2004;25(4):219-224.

112. Xu D, Emoto N, Giaid A, Slaughter C, Kaw S, deWit D, Yanagisawa M. ECE-1: a membrane-bound metalloprotease that catalyzes the proteolytic activation of big endothelin-1. *Cell.* 1994;78(3):473-485.

113. Emoto N, Yanagisawa M. Endothelin-converting enzyme-2 is a membrane-bound, phosphoramidon-sensitive metalloprotease with acidic pH optimum. *The Journal of biological chemistry.* 1995;270(25):15262-15268.

114. Fernandez-Patron C, Radomski MW, Davidge ST. Vascular matrix metalloproteinase-2 cleaves big endothelin-1 yielding a novel vasoconstrictor. *Circulation research.* 1999;85(10):906-911.

115. Wypij DM, Nichols JS, Novak PJ, Stacy DL, Berman J, Wiseman JS. Role of mast cell chymase in the extracellular processing of big-endothelin-1 to endothelin-1 in the perfused rat lung. *Biochemical pharmacology.* 1992;43(4):845-853.

116. Kahler J, Ewert A, Weckmuller J, Stobbe S, Mittmann C, Koster R, Paul M, Meinertz T, Munzel T. Oxidative stress increases endothelin-1 synthesis in human coronary artery smooth muscle cells. *Journal of cardiovascular pharmacology.* 2001;38(1):49-57.

117. Wahl JR, Goetsch NJ, Young HJ, Van Maanen RJ, Johnson JD, Pea AS, Brittingham A. Murine macrophages produce endothelin-1 after microbial stimulation. *Experimental biology and medicine (Maywood, N.J.* 2005;230(9):652-658.

118. Kawaguchi Y, Suzuki K, Hara M, Hidaka T, Ishizuka T, Kawagoe M, Nakamura H. Increased endothelin-1 production in fibroblasts derived from patients with systemic sclerosis. *Annals of the rheumatic diseases.* 1994;53(8):506-510.

119. Hynynen MM, Khalil RA. The vascular endothelin system in hypertension--recent patents and discoveries. *Recent patents on cardiovascular drug discovery.* 2006;1(1):95-108.

120. Honore JC, Fecteau MH, Brochu I, Labonte J, Bkaily G, D'Orleans-Juste P. Concomitant antagonism of endothelial and vascular smooth muscle cell ETB receptors for endothelin induces hypertension in the hamster. *American journal of physiology.* 2005;289(3):H1258-1264.

121. Murakoshi N, Miyauchi T, Kakinuma Y, Ohuchi T, Goto K, Yanagisawa M, Yamaguchi I. Vascular endothelin-B receptor system in vivo plays a

favorable inhibitory role in vascular remodeling after injury revealed by endothelin-B receptor-knockout mice. *Circulation.* 2002;106(15):1991-1998.

122. Dupuis J, Jasmin JF, Prie S, Cernacek P. Importance of local production of endothelin-1 and of the ET(B)Receptor in the regulation of pulmonary vascular tone. *Pulmonary pharmacology & therapeutics.* 2000;13(3):135-140.

123. Barton M, Cosentino F, Brandes RP, Moreau P, Shaw S, Luscher TF. Anatomic heterogeneity of vascular aging: role of nitric oxide and endothelin. *Hypertension.* 1997;30(4):817-824.

124. Goettsch W, Lattmann T, Amann K, Szibor M, Morawietz H, Munter K, Muller SP, Shaw S, Barton M. Increased expression of endothelin-1 and inducible nitric oxide synthase isoform II in aging arteries in vivo: implications for atherosclerosis. *Biochemical and biophysical research communications.* 2001;280(3):908-913.

125. Kumazaki T, Wadhwa R, Kaul SC, Mitsui Y. Expression of endothelin, fibronectin, and mortalin as aging and mortality markers. *Experimental gerontology.* 1997;32(1-2):95-103.

126. Maeda S, Tanabe T, Miyauchi T, Otsuki T, Sugawara J, Iemitsu M, Kuno S, Ajisaka R, Yamaguchi I, Matsuda M. Aerobic exercise training reduces plasma endothelin-1 concentration in older women. *J Appl Physiol.* 2003;95(1):336-341.

127. Ishihata A, Katano Y. Role of angiotensin II and endothelin-1 receptors in aging-related functional changes in rat cardiovascular system. *Annals of the New York Academy of Sciences.* 2006;1067:173-181.

128. Struck J, Morgenthaler NG, Bergmann A. Proteolytic processing pattern of the endothelin-1 precursor in vivo. *Peptides.* 2005;26(12):2482-2486.

129. Papassotiriou J, Morgenthaler NG, Struck J, Alonso C, Bergmann A. Immunoluminometric assay for measurement of the C-terminal endothelin-1 precursor fragment in human plasma. *Clinical chemistry.* 2006;52(6):1144-1151.

130. Habib A, Al-Omari MA, Khaleghi M, Morgenthaler NG, Struck J, Bergmann A, Mosley TH, Turner ST, Kullo IJ. Plasma C-terminal pro-endothelin-1 is associated with target-organ damage in African Americans with hypertension. *American journal of hypertension.*23(11):1204-1208.

131. Jankowska EA, Filippatos GS, von Haehling S, Papassotiriou J, Morgenthaler NG, Cicoira M, Schefold JC, Rozentryt P, Ponikowska B, Doehner W, Banasiak W, Hartmann O, Struck J, Bergmann A, Anker SD, Ponikowski P. Identification of chronic heart failure patients with a high 12-month mortality risk using biomarkers including plasma C-terminal pro-endothelin-1. *PloS one.*6(1):e14506.

132. Khan SQ, Dhillon O, Struck J, Quinn P, Morgenthaler NG, Squire IB, Davies JE, Bergmann A, Ng LL. C-terminal pro-endothelin-1 offers additional prognostic information in patients after acute myocardial infarction: Leicester Acute Myocardial Infarction Peptide (LAMP) Study. *American heart journal.* 2007;154(4):736-742.

133. Van Guilder GP, Westby CM, Greiner JJ, Stauffer BL, DeSouza CA. Endothelin-1 vasoconstrictor tone increases with age in healthy men but can be reduced by regular aerobic exercise. *Hypertension.* 2007;50(2):403-409.

134. Rondaij MG, Bierings R, Kragt A, van Mourik JA, Voorberg J. Dynamics and plasticity of Weibel-Palade bodies in endothelial cells. *Arteriosclerosis, thrombosis, and vascular biology.* 2006;26(5):1002-1007.

135. Morawietz H, Talanow R, Szibor M, Rueckschloss U, Schubert A, Bartling B, Darmer D, Holtz J. Regulation of the endothelin system by shear stress in human endothelial cells. *The Journal of physiology.* 2000;525 Pt 3:761-770.

136. Xu H, Duan J, Wang W, Dai S, Wu Y, Sun R, Ren J. Reactive oxygen species mediate oxidized low-density lipoprotein-induced endothelin-1 gene expression via extracellular signal-regulated kinase in vascular endothelial cells. *Journal of hypertension.* 2008;26(5):956-963.

137. Yoshizumi M, Kurihara H, Morita T, Yamashita T, Oh-hashi Y, Sugiyama T, Takaku F, Yanagisawa M, Masaki T, Yazaki Y. Interleukin 1 increases the production of endothelin-1 by cultured endothelial cells. *Biochemical and biophysical research communications.* 1990;166(1):324-329.

138. Hai E, Ikura Y, Naruko T, Shirai N, Yoshimi N, Kayo S, Sugama Y, Fujino H, Ohsawa M, Tanzawa K, Yokota T, Ueda M. Alterations of endothelin-converting enzyme expression in early and advanced stages of human coronary atherosclerosis. *International journal of molecular medicine.* 2004;13(5):649-654.

139. Ihling C, Bohrmann B, Schaefer HE, Technau-Ihling K, Loeffler BM. Endothelin-1 and endothelin converting enzyme-1 in human atherosclerosis--novel targets for pharmacotherapy in atherosclerosis. *Current vascular pharmacology.* 2004;2(3):249-258.

140. Chung AW, Booth AD, Rose C, Thompson CR, Levin A, van Breemen C. Increased matrix metalloproteinase 2 activity in the human internal mammary artery is associated with ageing, hypertension, diabetes and kidney dysfunction. *Journal of vascular research.* 2008;45(4):357-362.

141. Bourque SL, Davidge ST, Adams MA. The interaction between endothelin-1 and nitric oxide in the vasculature: new perspectives. *Am J Physiol Regul Integr Comp Physiol.* 2011;300(6):R1288-1295.

142. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature.* 1980;288(5789):373-376.

143. Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proceedings of the National Academy of Sciences of the United States of America.* 1987;84(24):9265-9269.

144. Moncada S, Higgs EA. The discovery of nitric oxide and its role in vascular biology. *British journal of pharmacology.* 2006;147 Suppl 1:S193-201.

145. Mustafa AK, Gadalla MM, Snyder SH. Signaling by gasotransmitters. *Science signaling.* 2009;2(68):re2.

146. Michel T, Feron O. Nitric oxide synthases: which, where, how, and why? *The Journal of clinical investigation.* 1997;100(9):2146-2152.

147. Ramzy D, Rao V, Tumiati LC, Xu N, Sheshgiri R, Miriuka S, Delgado DH, Ross HJ. Elevated endothelin-1 levels impair nitric oxide homeostasis through a PKC-dependent pathway. *Circulation.* 2006;114(1 Suppl):I319-326.

148. Ramzy D, Rao V, Tumiati LC, Xu N, Miriuka S, Delgado D, Ross HJ. Role of endothelin-1 and nitric oxide bioavailability in transplant-related vascular injury: comparative effects of rapamycin and cyclosporine. *Circulation.* 2006;114(1 Suppl):I214-219.

149. Bohm F, Settergren M, Pernow J. Vitamin C blocks vascular dysfunction and release of interleukin-6 induced by endothelin-1 in humans in vivo. *Atherosclerosis.* 2007;190(2):408-415.

150. Dammanahalli KJ, Sun Z. Endothelins and NADPH oxidases in the cardiovascular system. *Clinical and experimental pharmacology & physiology.* 2008;35(1):2-6.

151. Anggrahini DW, Emoto N, Nakayama K, Widyantoro B, Adiarto S, Iwasa N, Nonaka H, Rikitake Y, Kisanuki YY, Yanagisawa M, Hirata K. Vascular endothelial cell-derived endothelin-1 mediates vascular inflammation and neointima formation following blood flow cessation. *Cardiovascular research.* 2009;82(1):143-151.

152. Bacon CR, Cary NR, Davenport AP. Endothelin peptide and receptors in human atherosclerotic coronary artery and aorta. *Circulation research.* 1996;79(4):794-801.

153. Marsden PA, Brenner BM. Transcriptional regulation of the endothelin-1 gene by TNF-alpha. *The American journal of physiology.* 1992;262(4 Pt 1):C854-861.

154. Morawietz H, Duerrschmidt N, Niemann B, Galle J, Sawamura T, Holtz J. Augmented endothelial uptake of oxidized low-density lipoprotein in response to endothelin-1. *Clin Sci (Lond).* 2002;103 Suppl 48:9S-12S.

155. Niemann B, Rohrbach S, Catar RA, Muller G, Barton M, Morawietz H. Native and oxidized low-density lipoproteins stimulate endothelin-converting enzyme-1 expression in human endothelial cells. *Biochemical and biophysical research communications.* 2005;334(3):747-753.

156. Taner CB, Severson SR, Best PJ, Lerman A, Miller VM. Treatment with endothelin-receptor antagonists increases NOS activity in hypercholesterolemia. *J Appl Physiol.* 2001;90(3):816-820.

157. Hernandez-Perera O, Perez-Sala D, Navarro-Antolin J, Sanchez-Pascuala R, Hernandez G, Diaz C, Lamas S. Effects of the 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors, atorvastatin and simvastatin, on the expression of endothelin-1 and endothelial nitric oxide synthase in vascular endothelial cells. *The Journal of clinical investigation.* 1998;101(12):2711-2719.

158. Mraiche F, Cena J, Das D, Vollrath B. Effects of statins on vascular function of endothelin-1. *British journal of pharmacology.* 2005;144(5):715-726.

159. Murray DB, McMillan R, Brower GL, Janicki JS. ETA selective receptor antagonism prevents ventricular remodeling in volume-overloaded rats. *American journal of physiology.* 2009;297(1):H109-116.

160. Lee KJ, Kim MK, Park YH, Seol HJ, Lim JE, Lee JN, Oh MJ. Vascular endothelial growth factor induces endothelin-1 production via matrix metalloproteinase-2 rather than endothelin-converting enzyme-1. *Hypertens Pregnancy.* 2007;26(2):189-199.

161. Gross J, Lapiere CM. Collagenolytic activity in amphibian tissues: a tissue culture assay. *Proceedings of the National Academy of Sciences of the United States of America.* 1962;48:1014-1022.

162. Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. *Nature.* 2000;408(6809):239-247.

163. Okamoto T, Akaike T, Sawa T, Miyamoto Y, van der Vliet A, Maeda H. Activation of matrix metalloproteinases by peroxynitrite-induced protein S-glutathiolation via disulfide S-oxide formation. *The Journal of biological chemistry.* 2001;276(31):29596-29602.

164. Viappiani S, Nicolescu AC, Holt A, Sawicki G, Crawford BD, Leon H, van Mulligen T, Schulz R. Activation and modulation of 72kDa matrix metalloproteinase-2 by peroxynitrite and glutathione. *Biochemical pharmacology.* 2009;77(5):826-834.

165. Schulz R. Intracellular targets of matrix metalloproteinase-2 in cardiac disease: rationale and therapeutic approaches. *Annual review of pharmacology and toxicology.* 2007;47:211-242.

166. Frears ER, Zhang Z, Blake DR, O'Connell JP, Winyard PG. Inactivation of tissue inhibitor of metalloproteinase-1 by peroxynitrite. *FEBS letters.* 1996;381(1-2):21-24.

167. Sariahmetoglu M, Crawford BD, Leon H, Sawicka J, Li L, Ballermann BJ, Holmes C, Berthiaume LG, Holt A, Sawicki G, Schulz R. Regulation of matrix metalloproteinase-2 (MMP-2) activity by phosphorylation. *Faseb J.* 2007;21(10):2486-2495.

168. Tsuruda T, Kato J, Cao YN, Hatakeyama K, Masuyama H, Imamura T, Kitamura K, Asada Y, Eto T. Adrenomedullin induces matrix metalloproteinase-2 activity in rat aortic adventitial fibroblasts. *Biochemical and biophysical research communications.* 2004;325(1):80-84.

169. Woodrum DT, Ford JW, Cho BS, Hannawa KK, Stanley JC, Henke PK, Upchurch GR, Jr. Differential effect of 17-beta-estradiol on smooth muscle cell and aortic explant MMP2. *The Journal of surgical research.* 2009;155(1):48-53.

170. Fernandez-Patron C, Zouki C, Whittal R, Chan JS, Davidge ST, Filep JG. Matrix metalloproteinases regulate neutrophil-endothelial cell adhesion through generation of endothelin-1[1-32]. *Faseb J.* 2001;15(12):2230-2240.

171. McQuibban GA, Gong JH, Tam EM, McCulloch CA, Clark-Lewis I, Overall CM. Inflammation dampened by gelatinase A cleavage of monocyte chemoattractant protein-3. *Science (New York, N.Y.* 2000;289(5482):1202-1206.

172. Watts SW, Thakali K, Smark C, Rondelli C, Fink GD. Big ET-1 processing into vasoactive peptides in arteries and veins. *Vascular pharmacology.* 2007;47(5-6):302-312.

173. Fernandez-Patron C, Stewart KG, Zhang Y, Koivunen E, Radomski MW, Davidge ST. Vascular matrix metalloproteinase-2-dependent cleavage of calcitonin gene-related peptide promotes vasoconstriction. *Circulation research.* 2000;87(8):670-676.

174. Martinez A, Oh HR, Unsworth EJ, Bregonzio C, Saavedra JM, Stetler-Stevenson WG, Cuttitta F. Matrix metalloproteinase-2 cleavage of adrenomedullin produces a vasoconstrictor out of a vasodilator. *The Biochemical journal.* 2004;383(Pt. 3):413-418.

175. Chew DK, Conte MS, Khalil RA. Matrix metalloproteinase-specific inhibition of Ca2+ entry mechanisms of vascular contraction. *J Vasc Surg.* 2004;40(5):1001-1010.

176. Hao L, Du M, Lopez-Campistrous A, Fernandez-Patron C. Agonist-induced activation of matrix metalloproteinase-7 promotes vasoconstriction through the epidermal growth factor-receptor pathway. *Circulation research.* 2004;94(1):68-76.

177. Hao L, Nishimura T, Wo H, Fernandez-Patron C. Vascular responses to alpha1-adrenergic receptors in small rat mesenteric arteries depend on mitochondrial reactive oxygen species. *Arteriosclerosis, thrombosis, and vascular biology.* 2006;26(4):819-825.

178. Wang W, Schulze CJ, Suarez-Pinzon WL, Dyck JR, Sawicki G, Schulz R. Intracellular action of matrix metalloproteinase-2 accounts for acute myocardial ischemia and reperfusion injury. *Circulation.* 2002;106(12):1543-1549.

179. Sawicki G, Leon H, Sawicka J, Sariahmetoglu M, Schulze CJ, Scott PG, Szczesna-Cordary D, Schulz R. Degradation of myosin light chain in isolated rat hearts subjected to ischemia-reperfusion injury: a new intracellular target for matrix metalloproteinase-2. *Circulation.* 2005;112(4):544-552.

180. Ali MA, Cho WJ, Hudson B, Kassiri Z, Granzier H, Schulz R. Titin is a target of matrix metalloproteinase-2: implications in myocardial ischemia/reperfusion injury. *Circulation.* 2010;122(20):2039-2047.

181. Andrew PJ, Mayer B. Enzymatic function of nitric oxide synthases. *Cardiovascular research.* 1999;43(3):521-531.

182. MacMicking JD, Nathan C, Hom G, Chartrain N, Fletcher DS, Trumbauer M, Stevens K, Xie QW, Sokol K, Hutchinson N, et al. Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. *Cell.* 1995;81(4):641-650.

183. Xia Y, Roman LJ, Masters BS, Zweier JL. Inducible nitric-oxide synthase generates superoxide from the reductase domain. *The Journal of biological chemistry.* 1998;273(35):22635-22639.

184. Xia Y, Tsai AL, Berka V, Zweier JL. Superoxide generation from endothelial nitric-oxide synthase. A Ca2+/calmodulin-dependent and tetrahydrobiopterin regulatory process. *The Journal of biological chemistry.* 1998;273(40):25804-25808.

185. Gorren AC, Schrammel A, Schmidt K, Mayer B. Thiols and neuronal nitric oxide synthase: complex formation, competitive inhibition, and enzyme stabilization. *Biochemistry.* 1997;36(14):4360-4366.

186. Kleinert H, Wallerath T, Euchenhofer C, Ihrig-Biedert I, Li H, Forstermann U. Estrogens increase transcription of the human endothelial NO synthase

gene: analysis of the transcription factors involved. *Hypertension.* 1998;31(2):582-588.

187. Smiley DA, Khalil RA. Estrogenic compounds, estrogen receptors and vascular cell signaling in the aging blood vessels. *Current medicinal chemistry.* 2009;16(15):1863-1887.

188. Balligand JL. Heat shock protein 90 in endothelial nitric oxide synthase signaling: following the lead(er)? *Circulation research.* 2002;90(8):838-841.

189. Brouet A, Sonveaux P, Dessy C, Balligand JL, Feron O. Hsp90 ensures the transition from the early Ca2+-dependent to the late phosphorylation-dependent activation of the endothelial nitric-oxide synthase in vascular endothelial growth factor-exposed endothelial cells. *The Journal of biological chemistry.* 2001;276(35):32663-32669.

190. Pritchard KA, Jr., Ackerman AW, Gross ER, Stepp DW, Shi Y, Fontana JT, Baker JE, Sessa WC. Heat shock protein 90 mediates the balance of nitric oxide and superoxide anion from endothelial nitric-oxide synthase. *The Journal of biological chemistry.* 2001;276(21):17621-17624.

191. Fleming I, Mohamed A, Galle J, Turchanowa L, Brandes RP, Fisslthaler B, Busse R. Oxidized low-density lipoprotein increases superoxide production by endothelial nitric oxide synthase by inhibiting PKCalpha. *Cardiovascular research.* 2005;65(4):897-906.

192. Brouet A, Sonveaux P, Dessy C, Moniotte S, Balligand JL, Feron O. Hsp90 and caveolin are key targets for the proangiogenic nitric oxide-mediated effects of statins. *Circulation research.* 2001;89(10):866-873.

193. Sun J, Druhan LJ, Zweier JL. Dose dependent effects of reactive oxygen and nitrogen species on the function of neuronal nitric oxide synthase. *Archives of biochemistry and biophysics.* 2008;471(2):126-133.

194. Sun J, Druhan LJ, Zweier JL. Reactive oxygen and nitrogen species regulate inducible nitric oxide synthase function shifting the balance of nitric oxide and superoxide production. *Archives of biochemistry and biophysics.* 2010;494(2):130-137.

195. Santhanam L, Christianson DW, Nyhan D, Berkowitz DE. Arginase and vascular aging. *J Appl Physiol.* 2008;105(5):1632-1642.

196. Durante W, Johnson FK, Johnson RA. Arginase: a critical regulator of nitric oxide synthesis and vascular function. *Clinical and experimental pharmacology & physiology.* 2007;34(9):906-911.

197. Crabtree MJ, Channon KM. Synthesis and recycling of tetrahydrobiopterin in endothelial function and vascular disease. *Nitric Oxide.* 2011.

198. Yang YM, Huang A, Kaley G, Sun D. eNOS uncoupling and endothelial dysfunction in aged vessels. *American journal of physiology.* 2009;297(5):H1829-1836.

199. Presta A, Siddhanta U, Wu C, Sennequier N, Huang L, Abu-Soud HM, Erzurum S, Stuehr DJ. Comparative functioning of dihydro- and tetrahydropterins in supporting electron transfer, catalysis, and subunit dimerization in inducible nitric oxide synthase. *Biochemistry.* 1998;37(1):298-310.

200. Schwarz PM, Kleinert H, Forstermann U. Potential functional significance of brain-type and muscle-type nitric oxide synthase I expressed in adventitia and media of rat aorta. *Arteriosclerosis, thrombosis, and vascular biology.* 1999;19(11):2584-2590.

201. Boulanger CM, Heymes C, Benessiano J, Geske RS, Levy BI, Vanhoutte PM. Neuronal nitric oxide synthase is expressed in rat vascular smooth muscle cells: activation by angiotensin II in hypertension. *Circulation research.* 1998;83(12):1271-1278.

202. Brophy CM, Knoepp L, Xin J, Pollock JS. Functional expression of NOS 1 in vascular smooth muscle. *American journal of physiology.* 2000;278(3):H991-997.

203. Bachetti T, Comini L, Curello S, Bastianon D, Palmieri M, Bresciani G, Callea F, Ferrari R. Co-expression and modulation of neuronal and endothelial nitric oxide synthase in human endothelial cells. *Journal of molecular and cellular cardiology.* 2004;37(5):939-945.

204. Huang A, Sun D, Shesely EG, Levee EM, Koller A, Kaley G. Neuronal NOS-dependent dilation to flow in coronary arteries of male eNOS-KO mice. *American journal of physiology.* 2002;282(2):H429-436.

205. Rand MJ, Li CG. Nitric oxide as a neurotransmitter in peripheral nerves: nature of transmitter and mechanism of transmission. *Annual review of physiology.* 1995;57:659-682.

206. Stamler JS, Meissner G. Physiology of nitric oxide in skeletal muscle. *Physiological reviews.* 2001;81(1):209-237.

207. Capettini LS, Cortes SF, Gomes MA, Silva GA, Pesquero JL, Lopes MJ, Teixeira MM, Lemos VS. Neuronal nitric oxide synthase-derived hydrogen peroxide is a major endothelium-dependent relaxing factor. *American journal of physiology.* 2008;295(6):H2503-2511.

208. Capettini LS, Cortes SF, Lemos VS. Relative contribution of eNOS and nNOS to endothelium-dependent vasodilation in the mouse aorta. *European journal of pharmacology.* 2010;643(2-3):260-266.

209. Stuehr D, Pou S, Rosen GM. Oxygen reduction by nitric-oxide synthases. *The Journal of biological chemistry.* 2001;276(18):14533-14536.

210. Tsai P, Weaver J, Cao GL, Pou S, Roman LJ, Starkov AA, Rosen GM. L-arginine regulates neuronal nitric oxide synthase production of superoxide and hydrogen peroxide. *Biochemical pharmacology.* 2005;69(6):971-979.

211. Weaver J, Porasuphatana S, Tsai P, Pou S, Roman LJ, Rosen GM. A comparative study of neuronal and inducible nitric oxide synthases: generation of nitric oxide, superoxide, and hydrogen peroxide. *Biochimica et biophysica acta.* 2005;1726(3):302-308.

212. Seddon MD, Chowienczyk PJ, Brett SE, Casadei B, Shah AM. Neuronal nitric oxide synthase regulates basal microvascular tone in humans in vivo. *Circulation.* 2008;117(15):1991-1996.

213. Seddon M, Melikian N, Dworakowski R, Shabeeh H, Jiang B, Byrne J, Casadei B, Chowienczyk P, Shah AM. Effects of neuronal nitric oxide synthase on human coronary artery diameter and blood flow in vivo. *Circulation.* 2009;119(20):2656-2662.

214. Kone BC, Kuncewicz T, Zhang W, Yu ZY. Protein interactions with nitric oxide synthases: controlling the right time, the right place, and the right amount of nitric oxide. *Am J Physiol Renal Physiol.* 2003;285(2):F178-190.

215. Song Y, Zweier JL, Xia Y. Heat-shock protein 90 augments neuronal nitric oxide synthase activity by enhancing Ca2+/calmodulin binding. *The Biochemical journal.* 2001;355(Pt 2):357-360.

216. Gingerich S, Krukoff TL. Activation of ERbeta increases levels of phosphorylated nNOS and NO production through a Src/PI3K/Akt-

dependent pathway in hypothalamic neurons. *Neuropharmacology.* 2008;55(5):878-885.

217. Chiueh C, Lee S, Andoh T, Murphy D. Induction of antioxidative and antiapoptotic thioredoxin supports neuroprotective hypothesis of estrogen. *Endocrine.* 2003;21(1):27-31.

218. Rosenfeld CR, Chen C, Roy T, Liu X. Estrogen selectively up-regulates eNOS and nNOS in reproductive arteries by transcriptional mechanisms. *Journal of the Society for Gynecologic Investigation.* 2003;10(4):205-215.

219. White RE, Han G, Dimitropoulou C, Zhu S, Miyake K, Fulton D, Dave S, Barman SA. Estrogen-induced contraction of coronary arteries is mediated by superoxide generated in vascular smooth muscle. *American journal of physiology.* 2005;289(4):H1468-1475.

220. White RE, Gerrity R, Barman SA, Han G. Estrogen and oxidative stress: A novel mechanism that may increase the risk for cardiovascular disease in women. *Steroids.* 2010;75(11):788-793.

CHAPTER 2:

COMBINED EFFECTS OF AGING AND OVARIAN DEFICIENCY RESULT IN INCREASED MMP-MEDIATED VASOCONSTRICTION

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Contribution: O Lekontseva (under the supervision of ST Davidge) designed the study, performed all the experiments and data analyses, and prepared the first draft of the manuscript. CF Rueda-Clausen assisted with ovariectomies. JS Morton provided complementary data (presented in Appendix A-2). All authors participated in a critical review of the manuscript.

2.1 INTRODUCTION

Both aging and estrogen deficiency are major constituents of the rise in CV risk associated with menopause. Understanding of the mechanisms involved in the vascular dysfunction attributable to either aging or ovarian withdrawal remains the subject of extensive experimentation. In the light of the recently admitted complexity of estrogen pathways under a variety of co-morbid vascular conditions, including aging,[1] atherosclerosis[2] and diabetes,[3] it has become increasingly important to understand molecular interactions between the constellations of risk factors. The focus of this study was to delineate the vascular impact of two independent risk factors, ovarian deficiency and aging, as well as their combination, as both conditions overlap in postmenopausal women and thereby pose unique challenges for CV risk management. In addition, a more complete and accurate description of animal models of menopause (for what either aged or Ovx animals were commonly used) is fundamental for acquiring mechanistic insights into the pathogenesis of vascular disease in female aging.

As detailed in Chapter 1 (section 1.5), MMPs are a family of proteolytic enzymes, activated in an inflammatory milieu and extensively involved in vascular tissue remodelling during the aging process.[4-6] MMPs have indeed the potential of regulating multiple vascular pathways due to their ability to degrade structural and signaling proteins such as the ECM, growth factors and cytokines, as well as proteins regulating vascular tone.[7] In particular, MMP may play a role in the vasoconstriction to bET-1, an MMP-2 substrate susceptible to proteolytic

degradation to a bioactive ET-1 fragment.[7] Whereas in normal conditions this pro-constrictor potential of MMP may remain quiescent, it may, hypothetically, become pronounced in aging where other, long term, MMP-mediated events take place. Indeed, thus far these enzymes have been primarily viewed as the chronic mediators of vascular remodelling, and their acute vasoactive role is less well understood.

Bioactive ET-1 peptide is the end-product of several posttranslational proteolytic steps. Its immediate precursor bET-1 can be exported from the endothelial cell and elicit local and systemic vascular effects. In fact, its circulating levels were found to be of a prognostic value in the assessment of CVD.[8] bET-1 reportedly exerts a weak direct, but potent indirect vasoconstrictor response following specific proteolysis to mature forms such as classical small $ET\text{-}1_{[1\text{-}21]}$, or extended-length, $ET\text{-}1_{[1\text{-}31]}$ and $ET\text{-}1_{[1\text{-}32]}$, the latter being an MMP-dependent process.[9] Whereas, specific *in vivo* roles of alternative ET-1 isoforms are less well understood, the availability of their "synthases", including MMP, may be a critical rate-limiting factor.

To understand the role of MMP in acute mechanisms of vasoconstriction in female aging, the study was designed to analyze vascular effects of each factor, ovarian deficiency and aging, comprehensively. We hypothesized that combined aging and ovarian hormone deficiency augment MMP-mediated vasoconstriction via the ET-1 pathway.

2.2 METHODS

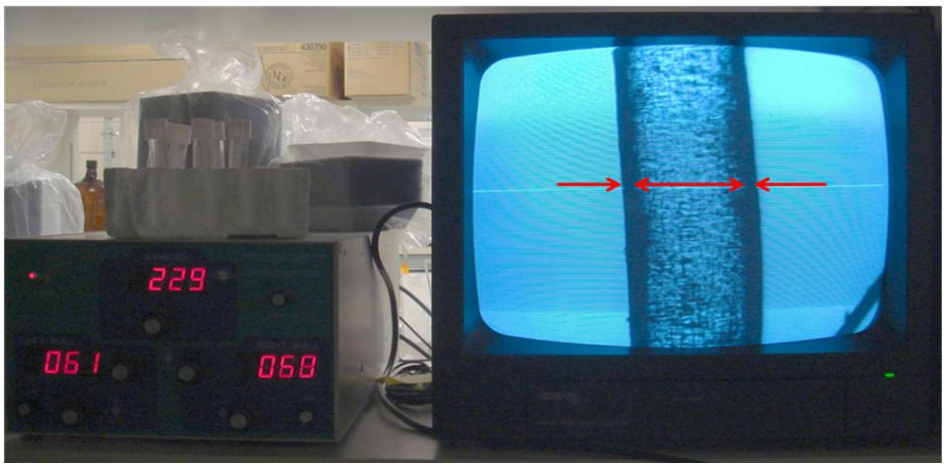
The experimental procedures were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee in accordance with the guidelines issued by the Canadian Council on Animal Care.

2.2.1 Experimental Animal Groups

Young and aged (3 and 12 months old) female Sprague Dawley rats were obtained from Charles River Breeding Laboratories (Quebec, Canada). Based on certain physiological characteristics, including the onset and cessation of reproductive cycling, these age groups were chosen as corresponding to young adult and older adult women (approximately 20 and 50 years old, respectively). Rats were housed in the animal facilities of the University of Alberta until experimentation. To identify independent vascular effects of two factors, ovarian deficiency and aging, young and aged rats were ovariectomized. All surgeries were conducted under inhaled isoflurane anaesthesia (5% induction, 2-3% maintenance). Both young Ovx and aged Ovx animals had respective age-matched controls with intact ovaries. After one month, rats were anesthetized with inhaled isoflurane, euthanized by exsanguination, and mesenteric arteries were collected. Blood samples were drawn from the chest cavity upon transection of the inferior vena cava, sera were prepared and stored at -80°C for subsequent assay of E2 (performed by the Capital Health Laboratory, University of Alberta Hospital).

2.2.2 Preparation of Isolated Vessels

A proximal segment of small intestine with attached mesentery was rapidly excised and immersed in ice-cold HEPES-buffered physiological saline solution (HEPES-PSS) of the following composition (mmol/l): 142 NaCl, 4.7 KCl, 1.17 $MgSO_4$, 1.56 $CaCl_2$, 1.18 KH_2PO_4, 10 HEPES, and 5.5 glucose (pH 7.5). Resistance-size arteries (200-250 μm inner diameter) were dissected free from adipose and connective tissue, cut into rings of 1-2 mm in length and transferred to a dual-chamber pressure arteriograph (Living Systems Instrumentation, Vermont). The lumen was gently flushed with HEPES-PSS to remove residual blood. The arteries were then secured with nylon ties between the glass microcannulae inside the chamber, where they were bathed in HEPES-PSS. Intraluminal pressure was gradually increased to 60 mmHg to approximate physiological values. Arterial dimensions, including wall thickness and inner diameter, were video monitored and measured using a video dimension analyzer (Living Systems Instrumentation, Vermont) as illustrated below.



2.2.3 Vascular Function Protocol

After a 30-min equilibration period in warm (37°C) HEPES-PSS (pH 7.4), arteries were pre-stretched by increasing intra-luminal pressure to 80 mmHg for 10 min. Pressure then was returned to 60 mmHg and maintained throughout the experimental protocol. Arteries were allowed to equilibrate for an additional 30 min. All the experiments were begun with a dose-response (doses added with a 2-min intervals) to phenylephrine (Phe, 0.01-10 μmol/l; Sigma) in the absence or presence of the MMP inhibitor, GM6001 (10 μmol/l; Calbiochem), which was applied to the vessel bath for 15 min prior to initiating the agonist-response curve. After completion of the curve, a 30-min recovery period was allowed, during which the baths were refilled every 10 min with the fresh HEPES-PSS. Arteries were again incubated with or without the MMP inhibitor, GM6001 (10 μmol/l) for 15 min, and assessed for dose-responses (doses added with a 5-min intervals) to bET-1 (0.001-1 μmol/l; Sigma). Dimethyl sulfoxide (DMSO), a vehicle for GM6001, was applied (1:1000 v/v) to the control vessel.

2.2.4 Vascular Protein Extraction

Frozen (-80°C) mesentery was thawed on ice, mesenteric arteries dissected and homogenized in 1 mol/l Tris-HCl buffer (pH 6.8) containing in mmol/l: 100 KCl, 0.5 $ZnCl_2$, 10 EDTA and 1% v/v Protease Inhibitor Cocktail (Sigma). The homogenate was centrifuged at 10,000 rpm for 10 min at 4°C, and the supernatant stored at -80°C. Arterial protein concentrations were determined by the bicinchoninic acid method using bovine serum albumin as a standard.

2.2.5 Gelatin Zymography

Gelatinolytic activities of MMP-2 and MMP-9 protein in the mesenteric vasculature were examined by gelatin zymography. Protein extracts from mesenteric arteries (9 µg protein/well) were loaded on 8% SDS polyacrylamide gels copolymerized with gelatin (2 mg/ml, type A, from porcine skin; Sigma). Following 1 hr of electrophoresis, the gels were washed with 2.5% Triton X-100 at room temperature for 1 hr, during which the solution was changed every 20 min. Gels were then incubated for 18 hrs at 37°C in incubation buffer: 50 Tris-HCl, 150 NaCl, 5 $CaCl_2$ (in mmol/l) and 0.05% NaN_3 (pH 7.6). After incubation, gels were stained with 0.05% Coomassie Brilliant Blue (G-250; Sigma) in a mixture of methanol: acetic acid: water (2.5:1:6.5 v/v) and de-stained in aqueous 4% methanol: 8% acetic acid (v/v). Gelatinolytic activities were detected as transparent bands against a dark blue background. Digital images were obtained with a high resolution scanner (Epson Expression 1680) and band intensities quantified using Quantity One software (V4.3.1; BioRad).

2.2.6 Statistical Analysis

The results are presented as means ± SEM. Dose-dependent changes in vascular diameter in response to Phe and bET-1 were expressed as percent vasoconstriction that was calculated using the formula: (1 - Dx/Dbaseline)*100, where Dx is a diameter at a given dose of agonist and Dbaseline is an initial vascular diameter. Resulting dose-response curves were fitted using nonlinear regression, and EC_{50} or area under the curve (AUC) were calculated. MMP

activities were expressed as a ratio to background intensity and normalized to the values observed in the control young intact group. Two-way ANOVA (with age and ovariectomy as two independent sources of variation) followed by Bonferroni's multiple comparison test was used to determine differences among the groups. All statistical analyses were performed using Graph Pad Prism V5.01 (Graph Pad Software Inc). Differences were considered statistically significant at $p<0.05$.

2.3 RESULTS

2.3.1 Animal Characteristics: E2 Levels, Body and Uterine Weights

Ovariectomy resulted in low serum E2 levels in both young Ovx (8.4 ±1.0 pg/ml) and aged Ovx (9.2 ±1.1 pg/ml) animals. In contrast, the circulating hormone levels in young intact female rats reflected the cycle phase (confirmed by a vaginal swab) with a mean of 26.4 ±4.5 pg/ml. There were variable concentrations of E2 in the blood samples from aged intact rats (which had entered the stage of continuous estrus), averaging 15.0 ±3.1 pg/ml (Table 2-1). Uterine weight, a biomarker of trophic stimulation by estrogen, was greatly reduced in hormone-depleted compared to intact rats at both age groups (Table 2-1).

2.3.2 Basal Conditions

The inner diameters of pressurized (60 mmHg) mesenteric arteries at the end of the equilibration period were not significantly different between all animal groups: 222 ±9 μm in young intact, 227 ±17 μm in young Ovx, 230 ±11 μm in aged intact, and 218 ±11 μm in aged Ovx; p=0.87 (one-way ANOVA).

2.3.3 Ovarian Deficiency Regardless of Age Increases Vasoconstriction to Big ET-1

We first examined reactivity of resistance arteries to exogenous bET-1. We found that Ovx animals of both age groups developed greater sensitivity to bET-1 compared to their age-matched controls. This was evidenced in the reduction of EC_{50} in young (Ovx 0.06 ±0.01 μmol/l vs. intact 0.21 ±0.02 μmol/l; p<0.001) or aged (Ovx 0.07 ±0.01 μmol/l vs. intact 0.15 ±0.03 μmol/l; p<0.01) groups (Figure 2-1).

2.3.4 Greater MMP Contribution to Vasoconstriction in Aging Ovx State

We next tested the MMP-dependent component in the bET-1, an MMP substrate, vasoactivity. MMP inhibition with GM6001 attenuated contractile effect of bET-1 in young Ovx (p<0.05), aged Ovx (p<0.001), and aged intact rats (p<0.05) (Figure 2-2B, C and D), but not young controls (Figure 2-2A). Interestingly, there was a greater attenuation of this constriction in the aged Ovx (reduction in AUC 3.8 ±0.6 mol/l x% $x10^{-5}$) compared to the young Ovx (1.5 ±0.5 mol/l x% $x10^{-5}$); p<0.05 or aged animals (1.8 ±0.6 mol/l x% $x10^{-5}$); p<0.05 (Figure

2-3). Unlike big ET-1, vascular response to ET-1 in aging rats was not affected by MMP inhibition (data shown in Appendix A-2).

Vascular sensitivity to the α-adrenergic agonist (Phe) was increased (p<0.05) by Ovx (EC_{50} = 0.44 ±0.13 μmol/l in young Ovx, and 0.45 ±0.05 μmol/l in aged Ovx) or aging (EC_{50} = 0.49 ±0.07 μmol/l in aged intact) relative to the young group (EC_{50} = 1.07 ± 0.18 μmol/l) (Figure 2-4). However, GM6001 did not have a significant effect on Phe-induced vasoconstriction in any of the animals (Figure 2-5A, B, C, and D).

2.3.5 Increased Vascular Tissue MMP-2 Activity in Aging Ovx State

To determine whether changes in vascular reactivity were associated with alterations in MMP activities, we used gelatin zymography to measure MMP-2 and -9 levels in the mesenteric vascular tissue. In aged Ovx animals only, we detected a pronounced increase (3.7-fold) in the cleaved, 66 kDa form of MMP-2 (p<0.05 vs. aged intact and p<0.001 vs. young Ovx) (Figure 2-6B), which was accompanied by a higher (1.4-fold) full-length, 72 kDa MMP-2 (p<0.01 vs. aged intact and p<0.001 vs. young Ovx) (Figure 2-6A). In the intact aged group, there was a similar trend to a higher 66 kDa (2.0-fold) MMP-2, an activated form of the enzyme; although no concomitant change in the 72 kDa form was evident (Figure 2-6A and B). In contrast to the impact of ovariectomy in aging, ovariectomy by itself (i.e. in young females) did not alter gelatinolytic activities in the vasculature (statistically, there was an interaction between the two factors, age and Ovx:

p<0.01 for 72kDa, *p*=0.07 for 66 kDa MMP-2 activity). MMP-9 (92 kDa) activity was barely detectable in the vascular tissue from all the animal groups.

2.4 DISCUSSION

In this study, we demonstrate that ovarian loss, irrespective of age, increases the sensitivity of arteries to bET-1. We also demonstrate that MMP inhibition attenuates this specific vasoconstrictor pathway, but has no effect on the vascular response to Phe. Interestingly, this pro-constrictor role of MMP is more pronounced in the hormone deficient aging condition (i.e. aged Ovx). This may be explained by elevated activity of MMP-2 measured in the aging arterial wall, which was further augmented by Ovx. In contrast, in young control animals, MMP inhibition did not have a significant functional effect, commensurate with the lower tissue MMP-2 levels and the overall low vascular reactivity to bET-1, which is only a weak agonist in its uncleaved form.

Study of CVD (such as hypertension) associated with female aging may be confounded by a limited suitability of animal models. Indeed, most of our basic understanding of CV pathways regulated by estrogen has derived from experimentation with male or Ovx female species, commonly of a young age. As directed by clinical needs in sex-specific CV medicine, there is now a growing awareness of interaction between the postmenopausal hormonal status and overlapping inflammatory vascular conditions (such as aging, hyperglycemia, hyperlipidemia, etc.). This concern has been raised in a number of recent

studies, as reviewed by Chakrabarti et al.[10] In fact, current hormone replacement research revisits the CV problem at the next level of complexity by differentiating between various hormone formulations,[11, 12] timing[13, 14] and routes of administration,[15] receptor selectivity[13] and specific vascular targets,[16, 17] taking into consideration a pre-developed with aging inflammatory background.[18]

Our present results did not show an age-related difference in the net vasoreactivity to exogenous bET-1, which was equally increased by Ovx at either age. This was surprising, since there has been substantial evidence of ET system activation during vascular aging.[19-21] Several other studies have suggested the ability of ovarian steroids (both estrogen and progesterone) to downregulate ET-1 synthesis[22-24] and modulate its vascular receptors,[12, 16] which is thought as an endothelial protective mechanism. Therefore, when disrupted in the Ovx condition, ET-1 may become a greater contributor to vascular dysfunction as a potent vasoconstrictor – as agrees with our present data (i.e. greater bET-1 sensitivity even in the young Ovx group), but also as an inflammatory cytokine and growth-promoting factor.[25, 26]

The role of MMP-2 in the specific enzymatic bET-1 conversion to ET-$1_{[1-32]}$ has been identified in the past studies conducted in our laboratory.[7] We now report that the MMP inhibitor (GM6001) attenuates bET-1-induced constriction in an Ovx state, with a greater effect in the aged Ovx. In the latter, the presence of higher mesenteric artery MMP-2 activity was confirmed by gelatin zymography. In contrast, we did not find a significant MMP role in the bET-1 response in arteries from young rats with intact ovarian function. These data support the possibility

that some of the deleterious effects of estrogen deficiency on vascular function can be linked to dysregulated MMPs, which may lead to increased alternative bET-1 processing. Moreover, in our past studies evaluating the age-associated vascular structural remodelling, we also observed alterations in MMP-2 activity in the aged rat female vasculature.[27] However, the physiologically relevant impact of Ovx was not known from the previous model.

Interestingly, a therapeutic strategy to oppose the actions of ET-1 has emerged, which targets ECE, the enzyme of the classical pathway leading to ET-$1_{[1-21]}$ production. Indeed, ECE inhibitors are being evaluated for potential benefits in vascular conditions such as essential hypertension.[28] Importantly, recent studies on the alternative cleavage product, ET-$1_{[1-31]}$, resulting from the activity of vascular chymase, suggested an enhanced role for this specific pathway in an experimental model of diabetes. Furthermore, Matsumoto et al.[29] documented sex differences in mesenteric artery reactivity to ET-$1_{[1-31]}$, which was pronounced in female but not male mice with chronic diabetes. This parallels our work on the other alternative ET-$1_{[1-32]}$ pathway mediated by MMP-dependent processing of bET-1, which functions in aging female rats (i.e. menopause model), but not aging male (data shown in Appendix A-1) or young animals. Thus, it is possible that in various vascular conditions one specific processing pathway can predominate. In perspective, the newly appreciated ET generating enzymes, including MMP, may harbor a therapeutic potential tailored to a specific vascular pathogenesis (e.g., postmenopausal vascular dysfunction). The latter case, if

proven, may provide a more specific alternative to postmenopausal hormone replacement.

Of note, in this study, no significant MMP role in the α-adrenergic constriction was evident in any of the animal groups, which allows excluding non-specific MMP effects on vasopressor mechanisms. Nonetheless, the vascular sensitivity to Phe was increased by the interventions (i.e. age, Ovx, or both) relative to baseline constriction elicited in intact young female rats. This age- or Ovx-related adrenergic sensitization has been previously described and may involve a number of mechanisms in postreceptor signaling.[30-33] Some investigators, however, propose MMP activation downstream in the adrenergic pathway as a pro-hypertensive mechanism that has been recently demonstrated in the rat mesenteric vasculature. In these reports, vasoconstrictive as well as growth-promoting effects of adrenergic (as well as other G protein-coupled receptors) agonists were attributed, in part, to MMP-mediated EGFR transactivation;[34, 35] however neither sex, nor age effects were assessed in this work. Given the G protein-coupled nature of ETRs too, it might be argued that MMP activity downstream (rather than upstream) of ET-1 might explain attenuated bET-1 response in the presence of GM6001. However, the fact that MMP inhibition affected only bET-1 but not ET-1 vasoconstriction in the aging female rats (Appendix A-2) supports the role of MMP in the processing of bET-1.

In conclusion, the mechanisms mediating sensitivity to vascular risk factors in female aging are not fully explained. Growing evidence implicates specific members of the MMP family in many aspects of vascular pathophysiology,

including earlier unknown acute vasomotor events. Our study suggests a newly identified MMP-mediated vasoconstrictor pathway that appears to play a role in an Ovx state in the aging females, thereby accounting, at least in part, for resistance artery dysfunction. This may undermine regulation of total peripheral resistance and thus have a major impact on systemic blood pressure, which rises even in clinically healthy women in postmenopausal period. Interestingly, mechanistically hormone deficiency by itself (i.e. without aging background) reveals a moderate pro-constrictor activity of MMP, which is otherwise absent. This becomes further pronounced in the aging process, where it is associated with greater vascular tissue MMP-2 activity. A better understanding of this previously unknown vasoconstrictor pathway in aging women may characterize a mechanism contributing to postmenopausal hypertension. Ultimately, potential non-hormonal therapeutic approaches, possibly involving specific MMP inhibition, may emerge.

2.5 REFERENCES

1. Stice JP, Lee JS, Pechenino AS, Knowlton AA. Estrogen, aging and the cardiovascular system. *Future cardiology.* 2009;5(1):93-103.

2. Umetani M, Domoto H, Gormley AK, Yuhanna IS, Cummins CL, Javitt NB, Korach KS, Shaul PW, Mangelsdorf DJ. 27-Hydroxycholesterol is an endogenous SERM that inhibits the cardiovascular effects of estrogen. *Nature medicine.* 2007;13(10):1185-1192.

3. Xu HL, Baughman VL, Pelligrino DA. Estrogen replacement treatment in diabetic ovariectomized female rats potentiates postischemic leukocyte

adhesion in cerebral venules. *Stroke; a journal of cerebral circulation.* 2004;35(8):1974-1978.

4. Jacob MP. Extracellular matrix remodeling and matrix metalloproteinases in the vascular wall during aging and in pathological conditions. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie.* 2003;57(5-6):195-202.

5. Kunz J. Matrix metalloproteinases and atherogenesis in dependence of age. *Gerontology.* 2007;53(2):63-73.

6. Schiffrin EL. Vascular stiffening and arterial compliance. Implications for systolic blood pressure. *American journal of hypertension.* 2004;17(12 Pt 2):39S-48S.

7. Fernandez-Patron C, Radomski MW, Davidge ST. Vascular matrix metalloproteinase-2 cleaves big endothelin-1 yielding a novel vasoconstrictor. *Circulation research.* 1999;85(10):906-911.

8. Masson S, Latini R, Anand IS, Barlera S, Judd D, Salio M, Perticone F, Perini G, Tognoni G, Cohn JN. The prognostic value of big endothelin-1 in more than 2,300 patients with heart failure enrolled in the Valsartan Heart Failure Trial (Val-HeFT). *Journal of cardiac failure.* 2006;12(5):375-380.

9. Watts SW, Thakali K, Smark C, Rondelli C, Fink GD. Big ET-1 processing into vasoactive peptides in arteries and veins. *Vascular pharmacology.* 2007;47(5-6):302-312.

10. Chakrabarti S, Lekontseva O, Davidge ST. Estrogen is a modulator of vascular inflammation. *IUBMB life.* 2008;60(6):376-382.

11. Mark CJ, Tatchum-Talom R, Martin DS, Eyster KM. Effects of estrogens and selective estrogen receptor modulators on vascular reactivity in the perfused mesenteric vascular bed. *American journal of physiology.* 2007;293(5):R1969-1975.

12. Pedersen SH, Nielsen LB, Pedersen NG, Nilas L, Ottesen B. Hormone therapy modulates ET(A) mRNA expression in the aorta of ovariectomised New Zealand White rabbits. *Gynecol Endocrinol.* 2009;25(3):175-182.

13. Pinna C, Cignarella A, Sanvito P, Pelosi V, Bolego C. Prolonged ovarian hormone deprivation impairs the protective vascular actions of estrogen receptor alpha agonists. *Hypertension.* 2008;51(4):1210-1217.

14. Suzuki S, Brown CM, Dela Cruz CD, Yang E, Bridwell DA, Wise PM. Timing of estrogen therapy after ovariectomy dictates the efficacy of its neuroprotective and antiinflammatory actions. *Proceedings of the National Academy of Sciences of the United States of America.* 2007;104(14):6013-6018.

15. Mueck AO, Genazzani AR, Samsioe G, Vukovic-Wysocki I, Seeger H. Low-dose continuous combinations of hormone therapy and biochemical surrogate markers for vascular tone and inflammation: transdermal versus oral application. *Menopause (New York, N.Y.* 2007;14(6):978-984.

16. Pedersen SH, Nielsen LB, Mortensen A, Nilas L, Ottesen B. Progestins oppose the effects of estradiol on the endothelin-1 receptor type B in coronary arteries from ovariectomized hyperlipidemic rabbits. *Menopause (New York, N.Y.* 2008;15(3):503-510.

17. Xu JW, Ikeda K, Yamori Y. Genistein inhibits expressions of NADPH oxidase p22phox and angiotensin II type 1 receptor in aortic endothelial cells from stroke-prone spontaneously hypertensive rats. *Hypertens Res.* 2004;27(9):675-683.

18. Jazbutyte V, Hu K, Kruchten P, Bey E, Maier SK, Fritzemeier KH, Prelle K, Hegele-Hartung C, Hartmann RW, Neyses L, Ertl G, Pelzer T. Aging reduces the efficacy of estrogen substitution to attenuate cardiac hypertrophy in female spontaneously hypertensive rats. *Hypertension.* 2006;48(4):579-586.

19. Ishihata A, Katano Y. Role of angiotensin II and endothelin-1 receptors in aging-related functional changes in rat cardiovascular system. *Annals of the New York Academy of Sciences.* 2006;1067:173-181.

20. Stauffer BL, Westby CM, DeSouza CA. Endothelin-1, aging and hypertension. *Current opinion in cardiology.* 2008;23(4):350-355.

21. Thijssen DH, Rongen GA, van Dijk A, Smits P, Hopman MT. Enhanced endothelin-1-mediated leg vascular tone in healthy older subjects. *J Appl Physiol.* 2007;103(3):852-857.

22. Bilsel AS, Moini H, Tetik E, Aksungar F, Kaynak B, Ozer A. 17Beta-estradiol modulates endothelin-1 expression and release in human endothelial cells. *Cardiovascular research.* 2000;46(3):579-584.

23. Dubey RK, Jackson EK, Keller PJ, Imthurn B, Rosselli M. Estradiol metabolites inhibit endothelin synthesis by an estrogen receptor-independent mechanism. *Hypertension.* 2001;37(2 Part 2):640-644.

24. Wilbert-Lampen U, Seliger C, Trapp A, Straube F, Plasse A. Female sex hormones decrease constitutive endothelin-1 release via endothelial sigma-1/cocaine receptors: an action independent of the steroid hormone receptors. *Endothelium.* 2005;12(4):185-191.

25. Anggrahini DW, Emoto N, Nakayama K, Widyantoro B, Adiarto S, Iwasa N, Nonaka H, Rikitake Y, Kisanuki YY, Yanagisawa M, Hirata K. Vascular endothelial cell-derived endothelin-1 mediates vascular inflammation and neointima formation following blood flow cessation. *Cardiovascular research.* 2009;82(1):143-151.

26. Bohm F, Pernow J. The importance of endothelin-1 for vascular dysfunction in cardiovascular disease. *Cardiovascular research.* 2007;76(1):8-18.

27. Zhang Y, Stewart KG, Davidge ST. Estrogen replacement reduces age-associated remodeling in rat mesenteric arteries. *Hypertension.* 2000;36(6):970-974.

28. Kirkby NS, Hadoke PW, Bagnall AJ, Webb DJ. The endothelin system as a therapeutic target in cardiovascular disease: great expectations or bleak house? *British journal of pharmacology.* 2008;153(6):1105-1119.

29. Matsumoto T, Kakami M, Kobayashi T, Kamata K. Gender differences in vascular reactivity to endothelin-1 (1-31) in mesenteric arteries from diabetic mice. *Peptides.* 2008;29(8):1338-1346.

30. Arenas IA, Armstrong SJ, Xu Y, Davidge ST. Tumor necrosis factor-alpha and vascular angiotensin II in estrogen-deficient rats. *Hypertension.* 2006;48(3):497-503.

31. Lam KK, Lee YM, Hsiao G, Chen SY, Yen MH. Estrogen therapy replenishes vascular tetrahydrobiopterin and reduces oxidative stress in ovariectomized rats. *Menopause (New York, N.Y.* 2006;13(2):294-302.

32. McKee AP, Van Riper DA, Davison CA, Singer HA. Gender-dependent modulation of alpha 1-adrenergic responses in rat mesenteric arteries. *Am J Physiol Heart Circ Physiol.* 2003;284(5):H1737-1743.

33. Stice JP, Eiserich JP, Knowlton AA. Role of aging versus the loss of estrogens in the reduction in vascular function in female rats. *Endocrinology.* 2009;150(1):212-219.

34. Hao L, Du M, Lopez-Campistrous A, Fernandez-Patron C. Agonist-induced activation of matrix metalloproteinase-7 promotes vasoconstriction through the epidermal growth factor-receptor pathway. *Circulation research.* 2004;94(1):68-76.

35. Fernandez-Patron C. Therapeutic potential of the epidermal growth factor receptor transactivation in hypertension: a convergent signaling pathway of vascular tone, oxidative stress, and hypertrophic growth downstream of vasoactive G-protein-coupled receptors? *Canadian journal of physiology and pharmacology.* 2007;85(1):97-104.

Table 2-1. Animal Characteristics

Group	Serum E2 (pg/ml)	Body weight (g)	Uterine weight (g)	Uterus/body weight (mg/g)
Young Intact	26.4 ±4.5	328.6 ±7.0	0.51 ±0.05	1.57 ±0.18
Young Ovx	8.4 ±1.0 *****	393.1 ±8.5 *****	0.12 ±0.01 *****	0.30 ±0.01 *****
Aged Intact	15.0 ±3.1 #	405.5 ±17.5 #	0.68 ±0.07 #	1.71 ±0.20
Aged Ovx	9.2 ±1.1	447.4 ±7.6 *****#	0.25 ±0.03 *****	0.55 ±0.05 *****

Data are presented as means ± SEM, n=5-10/group. Significant difference among the groups is indicated by symbols ***** Ovx vs. Intact, and # Aged vs. Young.

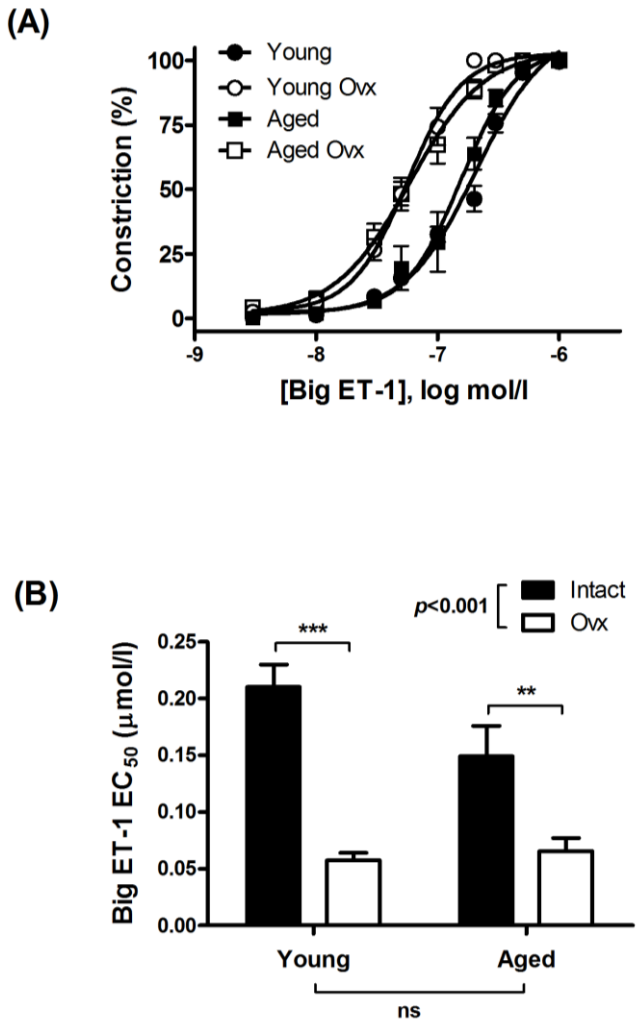


Figure 2-1. Ovarian deficiency regardless of age is associated with increased sensitivity to big ET-1

A: Dose-dependent constriction to big ET-1 in mesenteric arteries from young (solid circles), young Ovx (open circles), aged (solid squares) and aged Ovx (open squares) female rats. B: EC_{50} of big ET-1 responses. Data presented as means ± SEM, n=5-10/group. ***p<0.001, **p<0.01, ns=not significant.

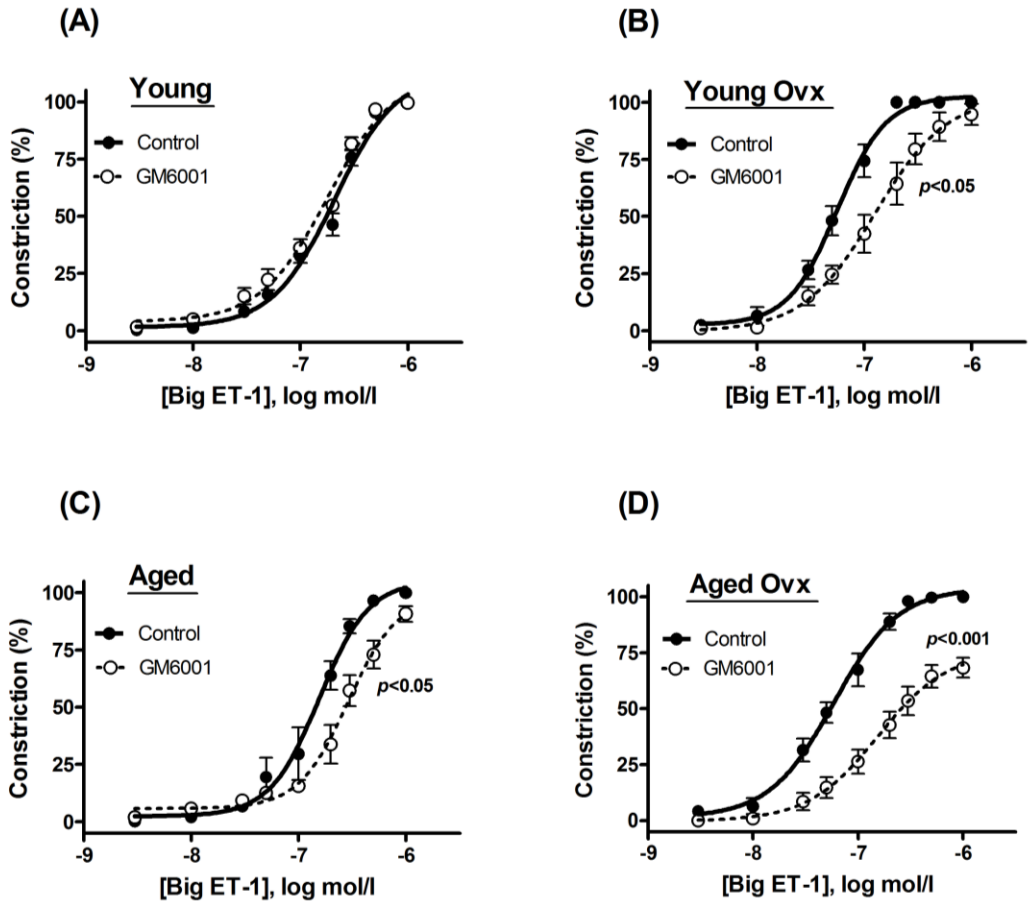


Figure 2-2. Pro-constrictior role of MMP is increased in aged Ovx state

Dose-dependent constriction of mesenteric arteries to big ET-1 in the absence (solid circles) or presence (open circles) of MMP inhibitor, GM6001 (10 µmol/l) in young (A), young Ovx (B), aged (C) and aged Ovx rats (D). Data presented as means ± SEM, n=5-10/group, *p* values are shown (control vs GM6001, two-way ANOVA).

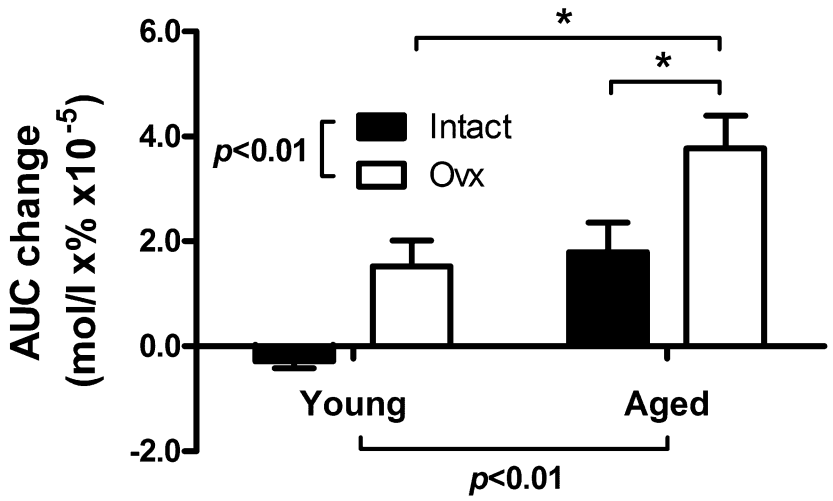


Figure 2-3. Additive effects of ageing and Ovx on a pro-constrictor potential of MMP, a summary graph

Change in AUC with MMP inhibition is greater in aged Ovx rats (*p<0.05). Data presented as means ± SEM, n=5-10/group.

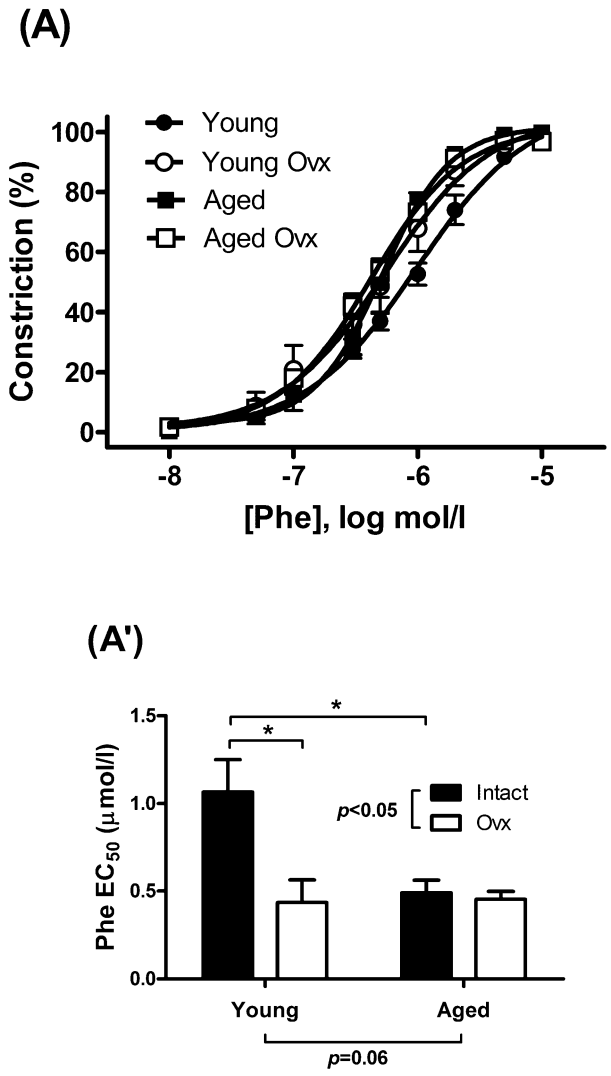


Figure 2-4. Sensitivity to adrenergic vasoconstriction also increases in aging or Ovx

A: Dose-dependent constriction of mesenteric arteries to phenylephrine in young (solid circles), young Ovx (open circles), aged (solid squares) and aged Ovx rats (open squares). A': EC_{50} of Phe responses. Data presented as means ± SEM, n=7-12/group. *p<0.05.

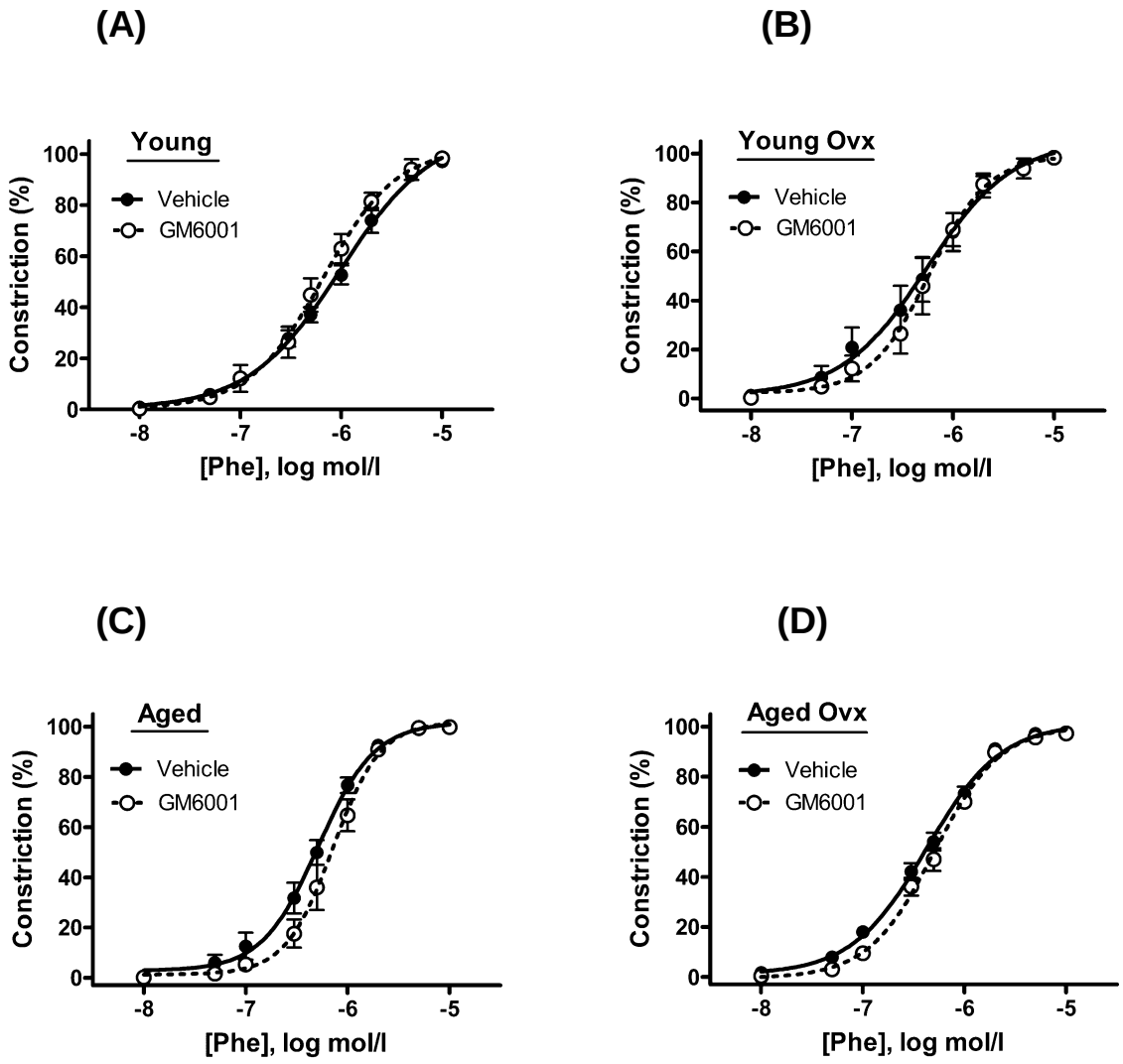


Figure 2-5. MMP is not involved in adrenergic vasoconstrictor mechanisms

Dose-responses to Phe are not altered in the presence of MMP inhibitor, GM6001 (open circles) compared to control vessels (solid circles) in any of the four experimental groups (A, B, C, and D). Data presented as means ± SEM (n=7-12/group).

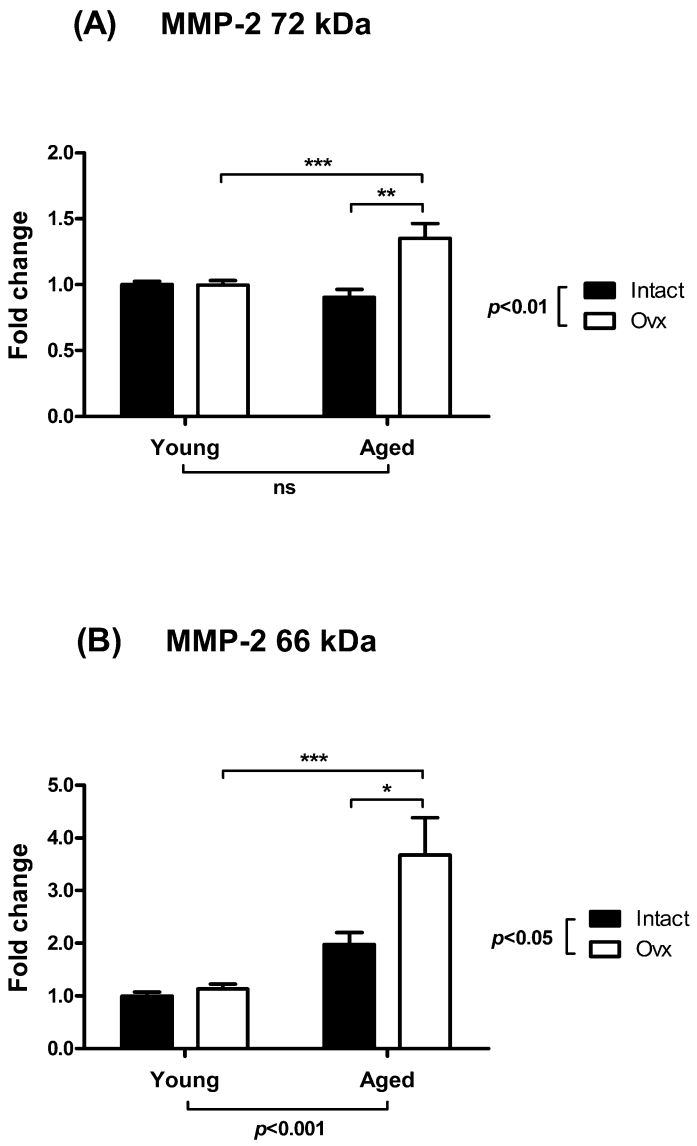


Figure 2-6. Greater vascular tissue MMP-2 activity in aging Ovx state

Full-length (A) and truncated/activated (B) MMP-2 in mesenteric arteries measured by gelatin zymography. Relative band intensities presented as means ± SEM of 4 independent experiments. *p<0.05, **p<0.01, ***p<0.001. Interaction between age and Ovx: p<0.01 (A) and p=0.07 (B).

CHAPTER 3:

ESTROGEN REPLACEMENT INCREASES MMP CONTRIBUTION TO VASOCONSTRICTION IN A MODEL OF MENOPAUSE

A version of this chapter has been published:
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Contribution: O Lekontseva (under the supervision of ST Davidge) designed the study, performed all the experiments (with assistance by Y Jiang in gelatin zymography) and data analyses, and prepared the first draft of the manuscript. All authors participated in a critical review of the manuscript.

3.1 INTRODUCTION

Menopause is a well established risk factor for CVD. Unique to female sex, age-related vascular dysfunction does not typically manifest at a clinical level until there is a decline in ovarian function and the circulating hormones, in particular estrogen. However, neither the interactions between the two risk factors (age and estrogen deficiency), nor the mechanisms of estrogen action in the vasculature compromised by aging are well understood.[1] The previous chapter (Chapter 2), described synergistic interactions between aging and ovarian hormone deficiency (that mimics menopause) in potentiating vasoconstrictor mechanisms. Although, estrogen replacement had been proposed for the CV risk reduction in postmenopausal women, its safety and efficacy remains a long-standing debate.[2, 3] Therefore, critical reconsideration and better understanding of pathways regulated by estrogen in the aging vasculature is needed.

As overviewed in the introductory chapter (section 1.4), vascular aging is associated with the development of subclinical tissue inflammation, characterized by elevated levels of pro-inflammatory cytokines such as TNF.[4] Numerous pro-inflammatory, pro-constrictor, pro-thrombotic pathways triggered downstream of TNF have been implicated in the pathogenesis of hypertensive vascular disease.[5, 6] TNF is known to induce MMPs,[7, 8] proteases involved in posttranslational modification of proteins, including vasoactive peptide bET-1, a precursor of vasoconstrictor compound ET-$1_{[1-32]}$.[9] While pathological ET-1 signaling is a characteristic feature of endothelial dysfunction associated with

aging,[10, 11] less is understood whether TNF or MMPs play a causative role. The latter, indeed, was shown to be a mechanism contributing to increased vasoconstriction in a model of female aging, whereas at a young age it did not have such a pathological role (data presented in Chapter 2). From the above study, however, the causes/triggers of acute pro-constrictor MMP activity were not clear, and the next series of experiments will examine TNF as a hypothetical upstream culprit.

The Davidge laboratory has previously demonstrated that E2 supplementation in aging animals improves vascular endothelial cell function, normalizes reactivity to α-adrenergic constriction and plays an anti-inflammatory role by reducing circulating bioactive TNF levels.[5, 12] This chapter will present results of the study testing whether rapid vascular effects of E2 downstream of TNF are mediated by MMP, and whether there is a role for this pathway in the regulation of vascular function in a model of menopause. In summary, we propose that TNF-induced MMP activity in the aged Ovx vasculature leads to a greater vasoconstriction through the ET-1 pathway. We then test the hypothesis that either E2 replacement or TNF inhibition will rescue vascular function to a similar extent by preventing excessive MMP activation.

3.2 METHODS

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The experimental procedures were approved by the

University of Alberta Health Sciences Animal Policy and Welfare Committee in accordance with the guidelines issued by the Canadian Council on Animal Care (NIH Animal Welfare Assurance Number is A5070-01).

3.2.1 Animal Model of Menopause

Female Sprague Dawley rats (3 and 12 months old) were obtained from Charles River Breeding Laboratories (Quebec, Canada) and housed in the University of Alberta animal facility until experimentation. Rats undergo reproductive senescence at near 12 months of age followed by a state of continuous estrus with variable circulating estrogen levels. Thus, to mimic physiology of aging in women (i.e. transition to menopause with permanent decline in ovarian function and hypoestrogenemia), 12 months old rats were ovariectomized.

3.2.2 Experimental Groups

At the time of ovariectomy, rats were assigned into three groups: (1) Meno+E2: Ovx rats treated with E2 (Innovative Research of America: 1.5 mg/pellet, 60-day release, subcutaneous implants), (2) Meno+Etan: Ovx rats treated with TNF inhibitor, Etanercept (0.4 mg/kg x 2/week, subcutaneous injections), or (3) Meno+Plac: Ovx rats administered placebo (placebo for E2 pellet or sterile water injection, which were combined for the final analysis as both controls yielded similar results). This animal model has been described and the

doses of all *in vivo* treatments justified in our previous studies.[12] Cycling adult rats (4 months old, in mixed cycle) served as a reference group.

After four weeks, rats were euthanized by exsanguination while under isoflurane anaesthesia. Blood samples were collected from the chest cavity, allowed to clot, and centrifuged at 3000 rpm for 10 minutes. The resulting sera were stored at -80°C for subsequent assay of E2 (performed by the Capital Health Laboratory, University of Alberta Hospital).

3.2.3 Preparation of Isolated Vessels

Small mesenteric arteries were dissected and prepared for testing on a pressure arteriograph system as described in Methods of Chapter 2.

3.2.4 Vascular Function Protocol

Vascular reactivity to exogenous bET-1 (0.001-1 µmol/L) in the absence or presence of MMP inhibitor, GM6001 (10 µmol/l) was assessed following the protocol described in Methods of Chapter 2. Similarly, all the experiments were begun with the dose-response to Phe (0.01-10 µmol/L) following a 15-min preincubation with 10 µmol/l GM6001 or its vehicle, DMSO (1:1000 v/v).

3.2.5 Vascular Protein Extraction

Vascular protein extraction and storage were as described in Methods of Chapter 2.

3.2.6 Gelatin Zymography

Gelatinolytic activities of MMP in the mesenteric vascular bed were examined by gelatin zymography as detailed in Methods of Chapter 2.

3.2.7 Statistical Analysis

The results are shown as mean ± SEM. Percent vasoconstriction was calculated using the formula: (1-Dx/Dbaseline)*100 as described in Methods of Chapter 2. Vasoconstrictor dose-response curves were fitted using nonlinear regression. Comparisons were made based on EC_{50} (the agonist concentration that elicited 50% of vascular response) or the maximal constriction elicited by the maximal chosen concentration of agonist (GraphPad Prism 5.01; GraphPad Software Inc). MMP activities were expressed as a ratio to background intensity and normalized to the values obtained in the reference group (adult cycling females). T-test, one- or two-way ANOVA with Bonferroni's post test were used as appropriate to determine difference among the groups. Differences were considered statistically significant at values of $p<0.05$.

3.3 RESULTS

3.3.1 Animal Model

Ovariectomy resulted in low serum E2 levels in animals treated with either placebo (9.0 ±1.1 pg/ml) or Etanercept (11.0 ±1.7 pg/ml). As anticipated, there was a significant increase in the circulating hormone levels in the rats

administered exogenous E2 (63.8 ±16.4 pg/ml), which is comparable to the pro-estrus phase levels in the cycling females.

3.3.2 Basal Conditions

The inner diameters of pressurized (60 mmHg) mesenteric arteries at the end of equilibration period were not significantly different between all animal groups: 222 ±9 µm in Cycling, 235 ±8 µm in Meno+Plac, 223 ±11 µm in Meno+E2, and 218 ±14 µm in Meno+Etan.

3.3.3 Pro-constrictor Role for MMP in a Model of Menopause*

Resistance arteries isolated from "menopausal" rats (i.e. aged Ovx) were more sensitive to bET-1 compared to the cycling females (EC_{50}= 0.07 ±0.01 versus 0.21 ±0.02 µmol/l, respectively; $p<0.001$; Figure 3-1A). GM6001, a MMP inhibitor, attenuated bET-1 constriction in "menopausal" group (vehicle-treated vessels compared to GM6001-treated vessels: $p<0.001$, two-way ANOVA; Figure 3-1C). By contrast, in cycling animals, the vasoconstriction was not affected by MMP inhibition (Figure 3-1B). This observation suggests that in aging/estrogen deficiency, MMPs contribute to a pro-constrictor phenotype. Interestingly, response to bET-1 was not significantly altered in the presence of GM6001 in age-matched male counterparts (data shown in Appendix A-1), suggesting that the MMP-bET-1 pathway of vascular regulation might be unique to female vascular physiology.

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* *Figures 3-1 and 3-2 were partially shown in the context of Chapter 2.*

There was no measurable effect of MMP inhibition on α1-adrenergic constriction to Phe in either of the animal groups (Figure 3-2B and C), although sensitivity to the agonist, similarly, was greater in “menopausal” vasculature (EC_{50}=0.45 ±0.05 versus 1.07 ±0.18 μmol/l in menopausal and cycling groups, respectively; *p*=0.0015; Figure 3-2A).

3.3.4 Effect of E2 or anti-TNF Treatment on the MMP-Mediated Constriction

In agreement with the hypothesis, treatment with either E2 or Etanercept reduced vascular sensitivity to bET-1 (EC_{50}=0.07 ±0.01 in Meno+Plac vs. 0.15 ±0.04 in Meno+E2 or 0.16 ±0.03 μmol/l in Meno+Etan; $p<0.05$, one-way ANOVA; Figure 3-3). Although, MMP inhibition attenuated maximal constriction to bET-1 in arteries from all the three groups (Figure 3-4), there was a significantly greater ($p<0.01$, one-way ANOVA) effect in Meno+E2 group (reduction in max constriction=70.3 ±11.8%; Figure 3-4B) compared to Meno+Plac (36.1 ±5.2%; Figure 3-4A) or Meno+Etan groups (35.4 ±3.9%; Figure 3-4C).

3.3.5 Vascular Tissue MMP Activity in a Model of Menopause

To evaluate whether changes in vascular function were associated with alterations in MMP activity, we used gelatin zymography to measure gelatinase activity (MMP-2 and -9) in the mesenteric vasculature. In “menopausal” vasculature, MMP-2 activity was modestly increased at 72 kDa (1.2-fold) and further increased at 66 kDa (3.2-fold) compared to the activity found in control cycling rats (Figure 3-5). E2 or anti-TNF treatment reduced both pro- and active

forms of MMP-2 in the vascular tissue to the levels similar to that in the cycling group (Figure 3-5). MMP-9 activity (92 kDa band) was barely detectable in the samples from all the animal groups.

3.4 DISCUSSION

We examined efficacy of E2 treatment in reversing MMP-mediated vasoconstriction in a model of menopause. This piece of work extends observation of increased vascular reactivity to the ET-1 precursor, bET-1, which is acutely modulated by MMP in the state of "menopause" (reported in Chapter 2). Now, we show that supplemented E2 reduces sensitivity of arteries to bET-1, while strikingly, further increases MMP role in this vasoconstrictor pathway.

MMPs have long been viewed as key regulators of the long-term tissue remodelling in a variety of physiological and pathological conditions, owing to their ability to degrade proteins of the ECM.[13] Activity of vascular gelatinases can be amplified upon inflammatory stimulation, commonly present in aging.[7, 14] Indeed, we found increased MMP-2 activity in the mesenteric arteries from aged Ovx animals, which was restored by the treatment of animals with Etanercept or E2, both are known anti-inflammatory agents targeting TNF.[15] Altered activity of gelatinases underlies chronic vascular remodelling associated with hypertensive or atherosclerotic vascular disease.[16-18] Indeed, Davidge and other groups previously demonstrated that E2 treatment attenuates age-associated long-term vascular remodelling in estrogen-deficient females.[19, 20]

As a number of non-traditional, non-matrix MMP substrates in the vascular system were discovered during the recent decade,[21, 22] we were interested in understanding MMP-dependent posttranslational processing of bET-1 to ET-1, which might have a rapid effect on the vascular tone in conditions with altered MMP activity. Indeed, our data reveal substantial MMP contribution to the vasoactive mechanisms in a state of menopause. Likely, inflammatory milieu of aging together with increased oxidative stress in the vascular system leads to unbalanced MMP activity, which translates to altered regulation of vascular tone. Indeed, both inflammatory cytokines and ROS are potent regulators of MMPs, known to trigger both transcriptional and posttranslational mechanisms of enzyme activation.[23, 24] Interestingly, in other types of compromised states such as chronic hypoxia, which is frequently associated with CVD, induced MMP-2 activity also potentiates contractile responses in both conduit and resistance arteries.[25] It was speculated that the vasoregulatory role of MMP-2 might serve as a compensatory mechanism in the transition between alterations in vascular tone and structural remodelling in response to pathological stimuli.

Consistent with previous reports demonstrating beneficial effects of estrogen[26] or anti-TNF treatment[27] on vascular function, we also observed a decrease in vasoconstriction to bET-1 in the treated animals. In fact, the vascular responses in both treatment groups (E2 and Etanercept) were statistically similar, as we hypothesized given the previous characterization of TNF (by Davidge group) as a molecular target of estrogen in aging.[12] Moreover, restored vascular function resulting from either of the treatments was not significantly different from

the reference parameters in the cycling group. As an extension of the previous work, we now propose MMPs as a downstream mechanism of vascular dysfunction in the estrogen-TNF pathway. Although, based on the data we could speculate that improvement of the net vascular reactivity to bET-1 is a protective characteristic of E2, it is puzzling that MMP contribution to this constriction was not minimized to mimic control cycling rats. Moreover, it further increased relative to “menopausal” rats receiving placebo or Etanercept. This does not support TNF as a link between E2 and MMP activity in the vascular regulation, yet suggests a previously unknown role for estrogen as a regulator of vascular MMPs in aging. Molecular mechanisms mediating estrogen effects on MMP activity are poorly understood to date, and may involve both genomic mechanisms[28, 29] as well as, speculatively, posttranslational modulation of MMP activity via cross-talk with inflammatory and oxidative pathways.[2]

To reconcile reduced MMP-2 activity in the resistance vasculature of E2-replaced animals with greater MMP role in vascular function, it is important to understand that changes in the long-term MMP expression characteristic to aging phenotype may rather be reflective of on-going chronic remodelling processes, but not necessarily acute functional effects of the enzymes. Moreover, enzymatic activity localized to the endothelium may be the most important determinant for the acute functional effects on bET-1, which could have been masked with the gelatin zymography performed on the whole vessel homogenates. Yet on the other hand, identity of ECEs can encompass more proteases than is currently

known,[30] and therefore the specific MMP type implicated in bET-1 processing under the present experimental conditions will need to be further validated.

In summary, the mechanisms underlying pathophysiology of postmenopausal vascular alterations are complex, and implicate deficiency in ovarian estrogen superimposed with age-driven vascular changes. Our studies provided experimental evidence that MMP is a critical modulator of the vasoactive pathway that is not observed normally but aggravates vascular dysfunction in aging/Ovx state. E2 replacement appears to play a favorable role by attenuating sensitivity to bET-1. At the same time, this functional effect is accompanied by a pronounced involvement of MMP in the regulation of vascular tone, which was not evident in the control cycling animals. However, greater vasoactive role of MMP is not easily explained given the lower vascular tissue MMP-2 expression, emphasizing that the regulation of the enzymatic activity is more complex than can be measured by zymography. Taken together, our data suggest a previously unknown E2-MMP pathway of regulation of vascular function, which plays a role in a state of menopause and is not found in males. Understanding the mechanisms whereby E2 regulates MMPs warrants further investigation. A better knowledge of vascular effects of estrogens at fundamental level may allow its integration into practical approaches to reduce CVD associated with menopause.

3.5 REFERENCES

1. Reckelhoff JF, Fortepiani LA. Novel mechanisms responsible for postmenopausal hypertension. *Hypertension.* 2004;43(5):918-923.

2. Chakrabarti S, Lekontseva O, Davidge ST. Estrogen is a modulator of vascular inflammation. *IUBMB Life.* 2008;60(6):376-382.

3. Clark JH. A critique of Women's Health Initiative Studies (2002-2006). *Nucl Recept Signal.* 2006;4:e023.

4. De Martinis M, Franceschi C, Monti D, Ginaldi L. Inflamm-ageing and lifelong antigenic load as major determinants of ageing rate and longevity. *FEBS Letters.* 2005;579(10):2035-2039.

5. Arenas IA, Armstrong SJ, Xu Y, Davidge ST. Tumor necrosis factor-alpha and vascular angiotensin II in estrogen-deficient rats. *Hypertension.* 2006;48(3):497-503.

6. Vila E, Salaices M. Cytokines and vascular reactivity in resistance arteries. *Am J Physiol Heart Circ Physiol.* 2005;288(3):H1016-1021.

7. Siwik DA, Chang DL, Colucci WS. Interleukin-1beta and tumor necrosis factor-alpha decrease collagen synthesis and increase matrix metalloproteinase activity in cardiac fibroblasts in vitro. *Circ Res.* 2000;86(12):1259-1265.

8. Arenas IA, Xu Y, Lopez-Jaramillo P, Davidge ST. Angiotensin II-induced MMP-2 release from endothelial cells is mediated by TNF-alpha. *Am J Physiol Cell Physiol.* 2004;286(4):C779-784.

9. Fernandez-Patron C, Radomski MW, Davidge ST. Vascular matrix metalloproteinase-2 cleaves big endothelin-1 yielding a novel vasoconstrictor. *Circ Res.* 1999;85(10):906-911.

10. Stauffer BL, Westby CM, DeSouza CA. Endothelin-1, aging and hypertension. *Curr Opin Cardiol.* 2008;23(4):350-355.

11. Amiri F, Virdis A, Neves MF, Iglarz M, Seidah NG, Touyz RM, Reudelhuber TL, Schiffrin EL. Endothelium-restricted overexpression of human endothelin-1 causes vascular remodeling and endothelial dysfunction. *Circulation.* 2004;110(15):2233-2240.

12. Arenas IA, Armstrong SJ, Xu Y, Davidge ST. Chronic tumor necrosis factor-alpha inhibition enhances NO modulation of vascular function in estrogen-deficient rats. *Hypertension.* 2005;46(1):76-81.

13. Raffetto JD, Khalil RA. Matrix metalloproteinases and their inhibitors in vascular remodeling and vascular disease. *Biochem Pharmacol.* 2008;75(2):346-359.

14. Csiszar A, Ungvari Z, Koller A, Edwards JG, Kaley G. Aging-induced proinflammatory shift in cytokine expression profile in coronary arteries. *Faseb J.* 2003;17(9):1183-1185.

15. Reckelhoff JF. Cardiovascular disease, estrogen deficiency, and inflammatory cytokines. *Hypertension.* 2006;48(3):372-373.

16. Spiers JP, Kelso EJ, Siah WF, Edge G, Song G, McDermott BJ, Hennessy M. Alterations in vascular matrix metalloproteinase due to ageing and chronic hypertension: effects of endothelin receptor blockade. *Journal of hypertension.* 2005;23(9):1717-1724.

17. Wang M, Zhao D, Spinetti G, Zhang J, Jiang LQ, Pintus G, Monticone R, Lakatta EG. Matrix metalloproteinase 2 activation of transforming growth factor-beta1 (TGF-beta1) and TGF-beta1-type II receptor signaling within the aged arterial wall. *Arterioscler Thromb Vasc Biol.* 2006;26(7):1503-1509.

18. Libby P. The molecular mechanisms of the thrombotic complications of atherosclerosis. *J Intern Med.* 2008;263(5):517-527.

19. Zhang Y, Stewart KG, Davidge ST. Estrogen replacement reduces age-associated remodeling in rat mesenteric arteries. *Hypertension.* 2000;36(6):970-974.

20. Manson JE, Allison MA, Rossouw JE, Carr JJ, Langer RD, Hsia J, Kuller LH, Cochrane BB, Hunt JR, Ludlam SE, Pettinger MB, Gass M, Margolis KL, Nathan L, Ockene JK, Prentice RL, Robbins J, Stefanick ML. Estrogen therapy and coronary-artery calcification. *N Engl J Med.* 2007;356(25):2591-2602.

21. Fernandez-Patron C, Stewart KG, Zhang Y, Koivunen E, Radomski MW, Davidge ST. Vascular matrix metalloproteinase-2-dependent cleavage of calcitonin gene-related peptide promotes vasoconstriction. *Circ Res.* 2000;87(8):670-676.

22. Martinez A, Oh HR, Unsworth EJ, Bregonzio C, Saavedra JM, Stetler-Stevenson WG, Cuttitta F. Matrix metalloproteinase-2 cleavage of adrenomedullin produces a vasoconstrictor out of a vasodilator. *Biochem J.* 2004;383(Pt. 3):413-418.

23. Siwik DA, Colucci WS. Regulation of matrix metalloproteinases by cytokines and reactive oxygen/nitrogen species in the myocardium. *Heart Fail Rev.* 2004;9(1):43-51.

24. Schulz R. Intracellular targets of matrix metalloproteinase-2 in cardiac disease: rationale and therapeutic approaches. *Annu Rev Pharmacol Toxicol.* 2007;47:211-242.

25. He JZ, Quan A, Xu Y, Teoh H, Wang G, Fish JE, Steer BM, Itohara S, Marsden PA, Davidge ST, Ward ME. Induction of matrix metalloproteinase-2 enhances systemic arterial contraction after hypoxia. *Am J Physiol Heart Circ Physiol.* 2007;292(1):H684-693.

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

27. Csiszar A, Labinskyy N, Smith K, Rivera A, Orosz Z, Ungvari Z. Vasculoprotective effects of anti-tumor necrosis factor-alpha treatment in aging. *Am J Pathol.* 2007;170(1):388-398.

28. Wingrove CS, Garr E, Godsland IF, Stevenson JC. 17beta-oestradiol enhances release of matrix metalloproteinase-2 from human vascular smooth muscle cells. *Biochimica et biophysica acta.* 1998;1406(2):169-174.

29. Guccione M, Silbiger S, Lei J, Neugarten J. Estradiol upregulates mesangial cell MMP-2 activity via the transcription factor AP-2. *Am J Physiol Renal Physiol.* 2002;282(1):F164-169.

30. Tirapelli CR, Fecteau MH, Honore JC, Legros E, Gobeil F, D'Orleans-Juste P. Enzymatic pathways involved in the generation of endothelin-1(1-31) from exogenous big endothelin-1 in the rabbit aorta. *Br J Pharmacol.* 2006;148(4):527-535.

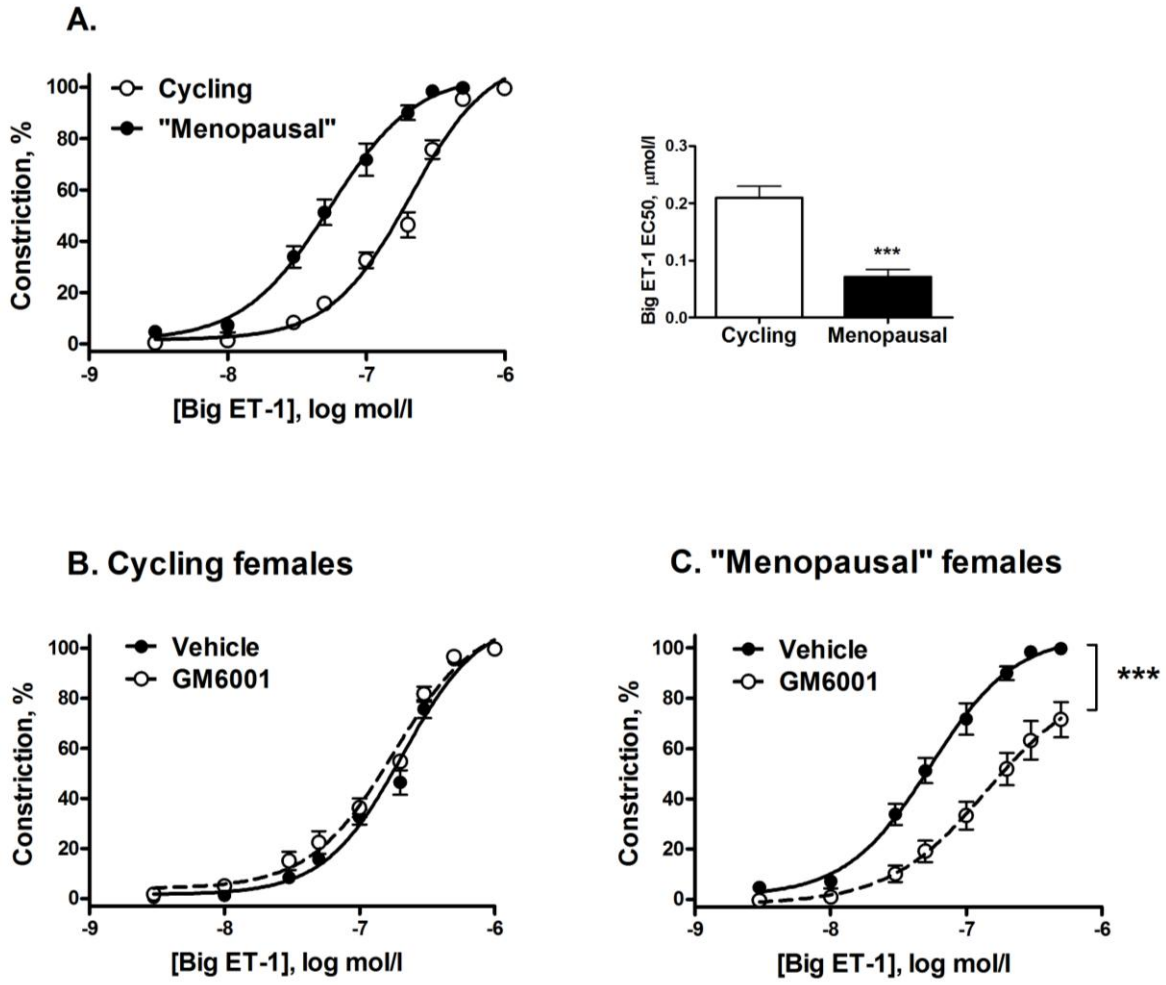

Figure 3-1. Greater bET-1 constriction in "menopause" is mediated by MMP

A: Big ET-1 dose-response (inset: EC_{50}) of mesenteric arteries from adult cycling (open circles; n=7) compared to "menopausal" female rat (solid circles; n=13). B and C: Big ET-1 dose-response in the absence (solid circles) or presence (open circles) of MMP inhibitor, GM6001 (10 µmol/l). Data presented as means ± SEM. ***p<0.001.

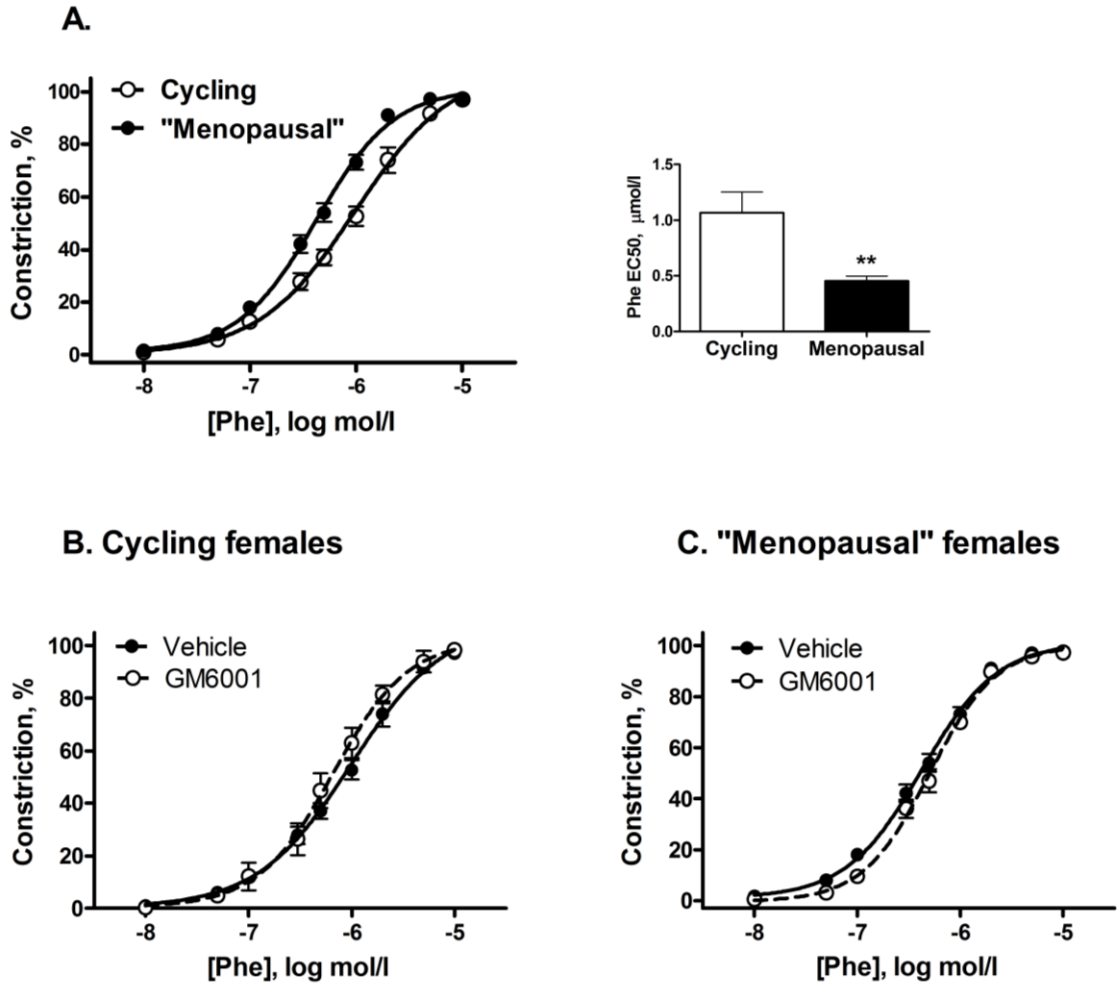

Figure 3-2. Adrenergic vasoconstriction is not mediated by MMP

A: Phe dose-response (inset: EC_{50}) of mesenteric arteries from adult cycling (open circles; n=14) compared to “menopausal” female rat (solid circles; n=16). Data presented as means ± SEM. ***p*<0.01. Phe-induced constriction was not altered in the presence of MMP inhibitor, GM6001 (open circles) compared to control vessel (solid circles) in either cycling (B) or menopausal group (C).

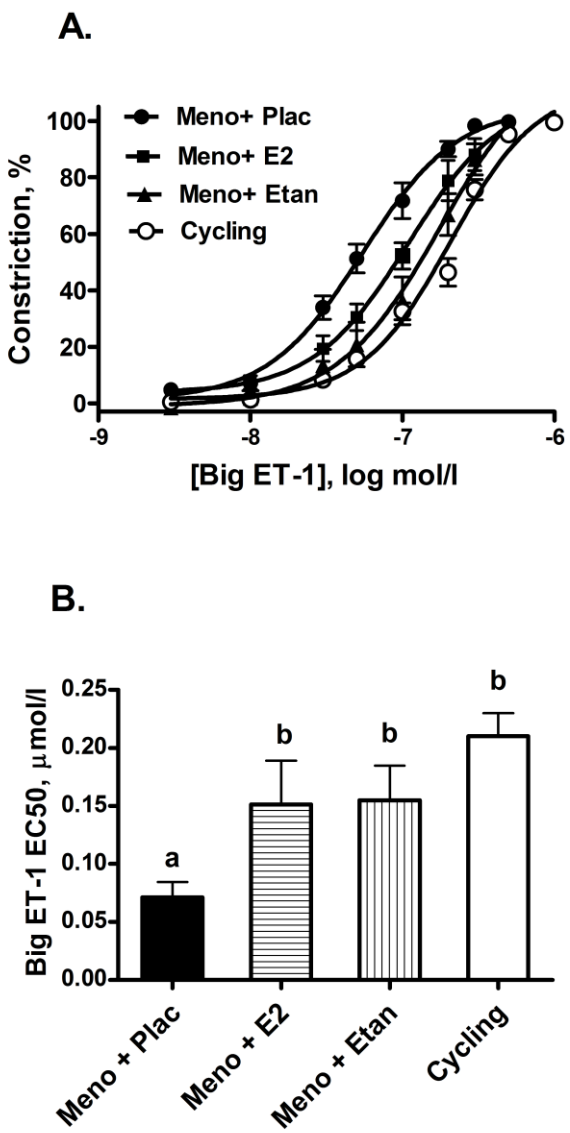

Figure 3-3. E2 or anti-TNF treatment attenuates big ET-1 sensitivity

A: Big ET-1 dose-response of mesenteric arteries from “menopausal” females treated for 4 weeks with placebo (solid circles; n=13), E2 (squares; n=9) or Etanercept (triangles; n=8) compared cycling females (open circles; n=7). B: EC_{50} of big ET-1 responses. Data presented as means ± SEM. $p<0.05$ a versus b.

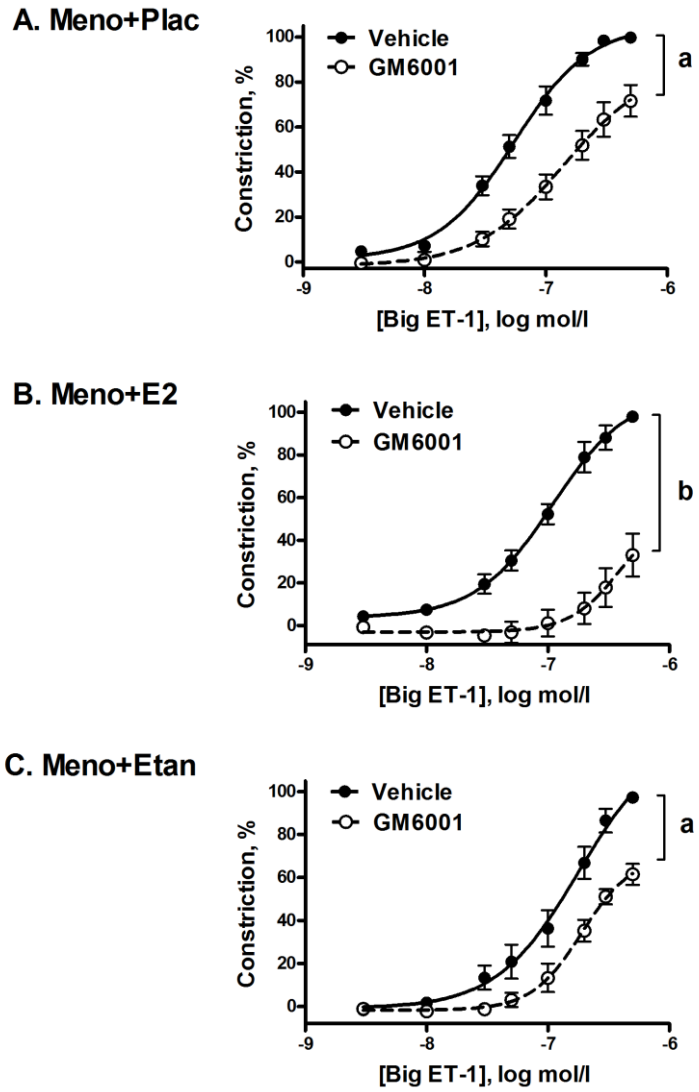

Figure 3-4. E2 replacement increases MMP contribution to vasoconstriction

Big ET-1 dose-response of mesenteric arteries in the absence (solid symbols) or presence (open symbols) of MMP inhibitor, GM6001 (10 μmol/l). Reduction of maximal vasoconstriction with MMP inhibition was greater in E2-treated (B) compared to placebo- (A) or Etanercept-treated rats (C). Data presented as means ± SEM, n=8-12 rats/group. $p<0.05$ a versus b.

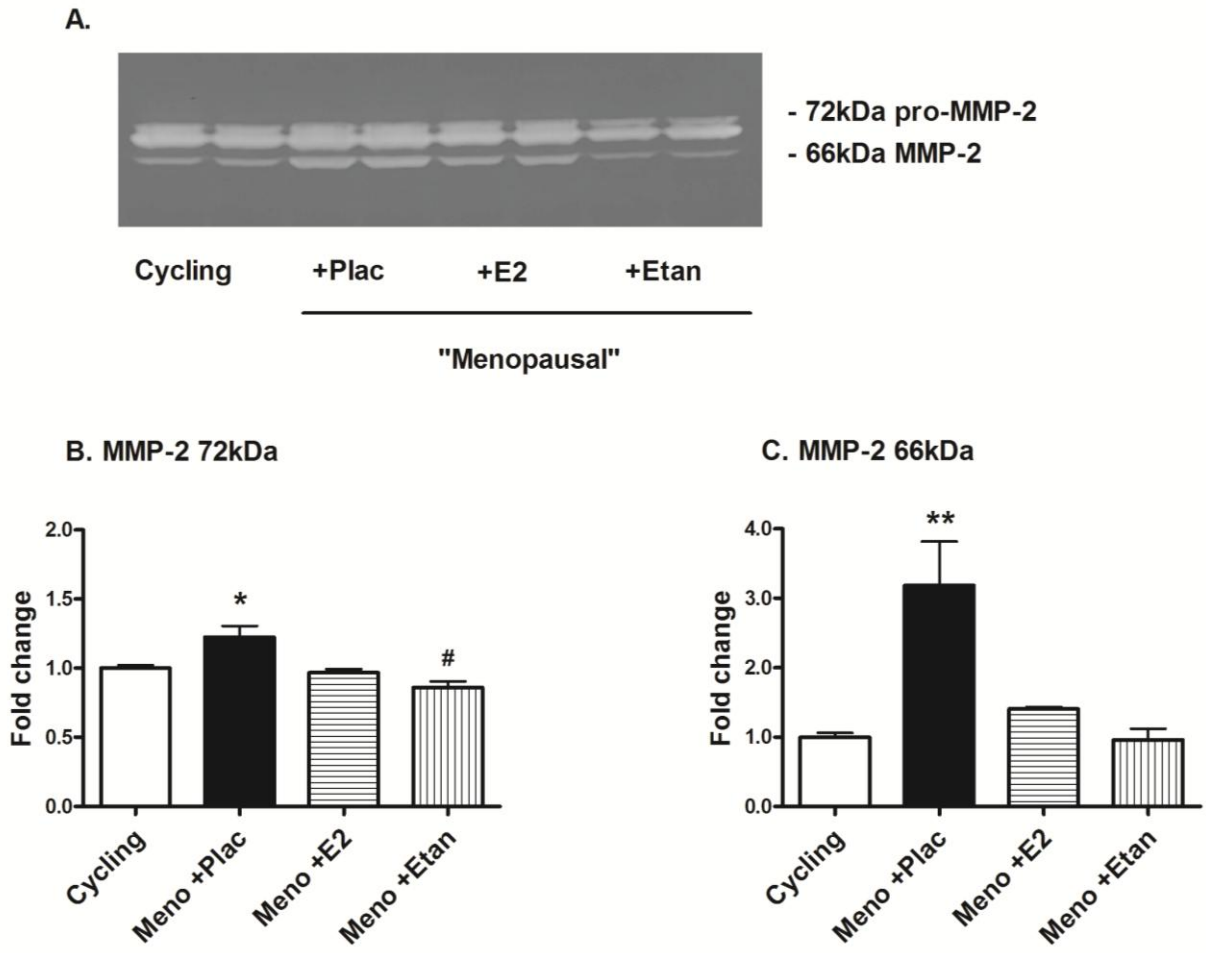

Figure 3-5. E2 replacement decreases long term vascular MMP expression

A: Representative gelatin zymogram showing MMP-2 activity in mesenteric arteries from cycling rats (lanes 1 and 2) and "menopausal" rats treated for 4 weeks with placebo (lanes 3 and 4), E2 (lanes 5 and 6), or Etanercept (lanes 7 and 8). B and C: ProMMP-2 (72 kDa) and active MMP-2 (66 kDa) shown as a fold change over the cycling rats. Data presented as means ± SEM of 4-6 independent experiments. *p<0.05 and **p<0.01 compared to cycling controls, $^{\#}p$<0.05 compared to "menopausal" rats treated with placebo.

CHAPTER 4:

NEURONAL NITRIC OXIDE SYNTHASE MEDIATES ESTROGEN-INDUCED RELAXATION IN RESISTANCE ARTERIES

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Contributions: O Lekontseva (together with S Chakrabarti and ST Davidge) designed the study, performed and analysed vessel function experiments, and prepared the first draft of the manuscript. S Chakrabarti (assisted by CC Cheung) performed and analysed cell culture experiments. Y Jiang performed vascular protein extraction and western blotting experiments. All authors participated in a critical review of the manuscript.

4.1 INTRODUCTION

The female sex hormone estrogen is involved in the regulation of many organ systems in both women and men. Estrogen regulation of key cardiometabolic parameters such as blood pressure is thought to contribute to sex-related differences in CV function in health and disease.[1] However, the molecular mechanisms and pathways of estrogen signaling in the vascular system are not sufficiently understood to allow for optimal clinical applications.

Estrogen, acting directly on vascular cells (e.g., endothelium and smooth muscle), has a vasorelaxant role.[2] Among a number of mechanisms (reviewed in Chapter 1), estrogen is known to modulate bioavailability of the local vasoactive factors. Studies presented in Chapters 2 and 3 were designed to assess the anti-vasoconstrictor actions of estrogen, focusing on the pathway leading to ET-1 generation. On the other hand, direct pro-vasorelaxant effects of estrogen are, in large part, thought to be mediated by the activation of eNOS, the enzyme catalyzing NO production in the vascular endothelium. As mentioned in section 1.6.2, estrogen via its plasma membrane associated receptors on endothelial cells acutely stimulates eNOS through the classical Ca^{2+}/CaM dependent mechanism involving the PI3K/Akt-mediated phosphorylation at Ser 1177.[3, 4] The resulting eNOS activity however is more complex, as other kinase pathways (such as, MAPK and Src) can be recruited to modulate eNOS phosphorylation status.[5, 6] This acute local regulation of eNOS function would translate into an immediate effect on vascular tone.

Interestingly, besides eNOS, another NOS isoform, nNOS, was found in endothelial cells in culture a few years ago.[7] Since then, other studies have identified it in the vascular wall in both experimental animals and humans.[8, 9] Although the functional significance of nNOS in vascular cells is poorly understood, a few studies suggested its role in mediating vascular relaxation in isolated arteries.[10, 11] Although nNOS and eNOS share some common characteristics (e.g., constitutive expression, Ca^{2+}-dependent activation and NO generation), they also possess unique properties and may have distinct roles in vascular function.[12-14] Similar to eNOS, a change in phosphorylation status determines nNOS enzymatic activity[15] – however, little is known about activating (at Ser 1417) versus inactivating (at Ser 847) regulatory phospho-sites in endothelial nNOS. Collectively, studies available to date suggest that nNOS may be involved in regulation of vascular function.

E2 was recently shown to activate nNOS via rapid Akt-dependent phosphorylation at the stimulatory site, Ser 1417, in isolated hypothalamic neurons[16] and human coronary artery smooth muscle cells;[17] with implications still remaining to be understood. The aim of this study was to test whether activity of nNOS in the vascular endothelium is regulated by E2. We hypothesized that E2 mediates its vasorelaxing effects, in part, via endothelial nNOS activation. We further examined differences in this pathway between sexes.

4.2 METHODS

4.2.1 Endothelial Cell Culture

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords obtained from term deliveries at the Royal Alexadra Hospital (Edmonton, Canada). Informed consent was obtained from all women prior to inclusion into this study. The protocol was approved by the University of Alberta Ethics Committee and the investigation conformed to the Declaration of Helsinki. We have previously used HUVEC as a well characterized widely used model to study human vascular endothelium. Important for the present study, HUVEC constitutively express both eNOS and nNOS, as well as classical ERs, ERα and ERβ (however, GPR30, another ER more recently shown in vascular cells, was below the level of detection in our HUVEC model). HUVEC isolation and culture were as previously described.[18] Briefly, the umbilical vein was flushed with PBS to remove blood clots, followed by HUVEC isolation using a type 1 collagenase-containing buffer. The cells were grown in a humidified atmosphere at 37°C with 5% CO_2/95% air in M199 medium with phenol red supplemented by 20% FBS as well as L-Glutamine (Gibko/ Invitrogen), Penicillin-Streptomycin (Life Technologies) and 1% ECGS. All experiments were conducted on second passage HUVEC. On the day of experiment, confluent monolayers of second passage HUVEC were quiesced in a Q-medium (phenol-free M199 medium with 1% FBS) for 4 hrs prior to treatments. For examining acute effects of estrogen on nNOS activity in the endothelium, we stimulated cells with E2 at 1 and 10 nmol/l

for 5 min. This is a physiological concentration range for endogenous estrogens, that has been shown before to induce eNOS activity within the chosen time frame, 5 min.[19] To determine whether acute effects of E2 on nNOS activity are mediated through the ERs, the cells were pre-treated with the classical ER blocker ICI 182780 (10 μmol/l) for 1 hr prior to 5 min stimulation with E2 at 10 and 100 nmol/l. At the end of the specified treatment period, the HUVEC were lysed in boiling hot Laemmli's buffer containing 0.2% Triton-X-100 to prepare samples for western blotting.

4.2.2 Western Blotting for Total and Phosphorylated nNOS

Western blotting was performed on the HUVEC lysates, which were prepared from equal number of cells in the same volume of the lysis buffer, followed by loading equal volumes of these lysates per well. The protein bands for phospho1417-nNOS and phospho847-nNOS (rabbit polyclonal antibodies from Abcam, 1μg/ml) were detected by a Fluor-S-Max multiimager and quantified by densitometry using Quantity One software (Bio-Rad). Data were normalized by re-probing the phospho-nNOS membranes with an antibody against total nNOS (mouse monoclonal antibody from BD Biosciences, 1μg/ml). Samples generated from a particular umbilical cord were run on the same gel. Cell lysates from untreated cells were loaded on every gel and all data were expressed as fold change over the corresponding untreated control.

4.2.3 Nitric Oxide Detection

Endothelial NO generation was determined by DAF-FM staining.[20] Confluent HUVEC monolayers were washed once and incubated with 10 μmol/l of DAF-FM in Q medium at room temperature for 30 min. To examine the effect of E2 on NO production, HUVEC were pre-treated with 10 nmol/l E2 for 30 min prior to application of DAF-FM. To assess the contribution of nNOS in E2-stimulated NO output, HUVEC were pre-treated with N-propyl-L-arginine (L-NPA, 2 μmol/l) the selective nNOS inhibitor, for 30 min prior to E2. After incubation with DAF-FM, cells were washed once and examined under a fluorescent microscope. Cell nuclei were visualized with Hoechst 33342 nuclear dye. For each data point, images from 3 randomly chosen fields were taken. The total fluorescence intensity and the number of cells in each field were found and the mean fluorescence intensity per cell (MFI/cell) was calculated. NO generation was determined as fold increase in MFI/cell over the untreated control (no E2 or nNOS inhibitor).

4.2.4 Animals

Sprague Dawley rats (3-4 months old) of both sexes were purchased from Charles River Breeding Laboratories (Quebec, Canada). The study was approved by the University of Alberta Animal Welfare Committee, and followed the Canadian Council on Animal Care and the US NIH guidelines for the Care and Use of Laboratory Animals.

4.2.5 Preparation of Isolated Vessels

Rats were euthanized by exsanguination under isoflurane anesthesia, and the mesentery was rapidly excised and placed in ice-cold HEPES-buffered PSS (as described in Chapter 2). Mesenteric arteries (~200 μm inner diameter) were carefully dissected out from surrounding tissue and cut into 2 mm-long segments.

4.2.6 nNOS Immunofluorescence on Vascular Sections

Mesenteric artery specimens, embedded in Tissue-Tek O.C.T Compound (Sakura Finetek), were frozen in liquid nitrogen and stored at -80°C. 10 μm-thick sections were placed on glass slides, dried overnight and stored at -80°C until experiment. Re-thawed vessel sections were fixed in acetone, incubated with blocking buffer (1% bovine serum albumin in PBS) and immunostained for 2 hr at room temperature with the anti-nNOS mouse monoclonal antibodies (BD Biosciences) in 1:250 dilution. Endothelial layer was visualized by co-immunostaining for von Willebrand's factor (rabbit polyclonal anti-vWF antibody from Chemicon, 1:400), an endothelial cell specific marker. Incubation with the secondary antibody (Alexa Fluor 546 (green) or 488 (red) from Molecular Probes) was done for 30 min in the dark. Glass coverslips were applied over the vessel sections with Vectashield H-1200 Mounting Kit (which also includes DAPI, a nuclear stain) (Vector Laboratories) and the slides were examined under an Olympus IX81 fluorescence microscope. Images were obtained using SlideBook imaging software and presented in x100 magnification. A control image, where no primary antibody, but the secondary antibody alone was applied, served to

detect any non-specific binding. The background auto-fluorescence was then subtracted from all the images, leaving the control images completely black, and only the true fluorescence from anti-nNOS or anti-vWF binding was visible.

4.2.7 *Ex Vivo* Vessel Function Assessment

Arterial rings were mounted in an isometric wire myograph system (Danish Myotechniques, Denmark) with two 40-μm wires threaded through the lumen. Vessels were bathed in 5 ml PSS, gassed with 5% CO_2/95% air, and maintained at a temparature of 37°C. Normalization of arteries to an optimal resting tension (set to 0.8 of IC_{100}, i.e. internal circumference equivalent to a transmural pressure of 100 mmHg) was conducted using LabChart7 software.

After a 30-min equilibration period, vessels were exposed twice to a 10 μmol/l dose of noradrenaline (NA) followed by a single 3 μmol/l dose of methacholine (MCh) to test for smooth muscle and endothelial integrity, respectively. A cumulative concentration-response curve (CCRC) to NA was then performed to determine the EC_{80} of the maximal response to the agonist. To investigate vascular response to E2 (0.001-10 μmol/l, doses added with a 2-min intervals) or MCh (0.003-3 μmol/l, doses added with a 2-min intervals), the CCRC was conducted following pre-constriction with the EC_{80} of NA. To assess the contribution of nNOS to E2- or MCh- induced vasorelaxation, the selective (L-NPA, 2 μmol/l) or non-selective (Nω-nitro-L-arginine methyl ester, L-NAME, 100 μmol/l) NOS inhibitor was applied to vessel bath for 30 min prior to pre-constriction. To determine the cell-specific source of nNOS, vascular responses

were also tested in endothelium-denuded arteries (achieved by threading human hair through the vessel lumen and confirmed by the absence of MCh-stimulated vasorelaxation).

4.2.8 Vascular Protein Extraction

Protein was extracted from thoracic aortas (both, male and female rats) and its concentration determined as described in Chapter 2. Samples were stored at -80°C until further measurement of NOS proteins by western blotting.

4.2.9 Western Blotting for NOS and ER proteins in Vascular Tissue

To examine sex-related differences in vascular protein expression, western blotting was performed on homogenized thoracic aortas isolated from male and female rats. Bands for eNOS and nNOS (mouse monoclonal antibodies from BD Biosciences, 1/250) were normalized to β-actin (rabbit polyclonal antibody from Abcam, 1/2000) and expressed as fold change male over female samples run on the same gel. Phospho1417- and phospho847-nNOS (rabbit polyclonal antibodies from Abcam, 1/400) were normalized to total nNOS. Bands obtained with anti-ERα and anti-ERβ (rabbit polyclonal antibodies from Santa Cruz, 1/200) were normalized to β-actin.

4.2.10 Statistical Analysis

Data are shown as mean ± SEM. One-way analysis of variance (ANOVA) followed by Bonferroni's test was used for comparisons in cell culture

experiments. Percent vascular relaxation to E2 and MCh were calculated using the formula: (Tpreconstricted - Tx)/(Tpreconstricted - Tbaseline)*100, where Tpreconstricted is a vessel wall tension (in mN) developed following application of NA EC_{80} from which the relaxation response was subsequently assessed, Tx is a tension at a given dose of agonist, and Tbaseline is a resting wall tension. Two-way repeated measures ANOVA (RMANOVA) with Bonferroni's post test was used to compare vascular responses to E2. MCh curves were fitted using non-linear regression, and EC_{50} were compared with one-way ANOVA followed by Bonferroni's test. NOS and ER expression in the male versus female rat aortas was compared with unpaired *t*-test. A p value < 0.05 was accepted as statistically significant.

4.3 RESULTS

4.3.1 E2 Acutely Activates nNOS and NO production in HUVECs

We found that stimulation of human endothelial cells with physiological doses of E2 (1 or 10 nmol/l) for 5 min increased nNOS activity. This was evidenced by greater phospho1417-nNOS presence, an activated form of nNOS, in E2-treated cells compared to untreated control HUVEC (Figure 4-1A). There was no change in the inhibitory phospho847-nNOS expression in the treated relative to untreated cells (Figure 4-1B). To note, anti-phosphoSer1417 and anti-phosphoSer847 detect multiple bands on the immunoblot, commonly a triple band for p1417-nNOS and a double band for p847-nNOS, the whole area of

which is quantified accordingly. E2-induced increase in phospho1417-nNOS was prevented in the cells pre-treated with the ER blocker, ICI 182780 (Figure 4-1C). Corresponding with the higher nNOS activity, there was an increased NO generation in E2-stimulated HUVEC. This was reflected by a greater intensity of DAF-FM fluorescence (1.4-fold, $p<0.01$) in the cells treated with E2 (10 nmol/l) compared to control HUVEC (Figure 4-2). Moreover, the E2-stimulated NO production was significantly attenuated in the cells pre-incubated with the nNOS inhibitor, L-NPA.

4.3.2 nNOS Expression in the Rat Mesenteric Artery

To confirm the presence of nNOS in whole vessels, we performed immunofluorescence using anti-nNOS antibody on cross-sections of mesenteric arteries from adult female rats. Strong nNOS-specific binding was detected in the endothelium (where it co-localized with anti-vWF) and adventitia, with weaker staining in the media (Figure 4-3).

4.3.3 nNOS Mediates Acute E2-Induced Relaxation in Mesenteric Arteries

In adult female rats, E2 elicited dose-dependent vascular relaxation of mesenteric arteries (26.2 ±3.7% at max dose), which was significantly ($p<0.001$) attenuated by pan-NOS inhibition with L-NAME, and also the specific nNOS inhibition with L-NPA (Figure 4-4A). In the endothelium-denuded arteries, E2 also elicited a relaxation response (19.8 ±2.3% at max dose), where the nNOS-dependent component was no longer observed (Figure 4-4B). This suggests that

predominantly endothelial source of nNOS contributes to E2 relaxation within the chosen concentration range.

4.3.4 Vascular Response to E2 in Male Rats Is NOS-Independent

Given that nNOS activity in the vascular system is regulated by E2, we tested whether there were sex-specific differences in this pathway. We observed reduced (p<0.01) vasorelaxation to exogenous E2 in the arteries isolated from male (6.4 ±2.9% at max dose) compared to female rats (26.2 ±3.7% at max dose) (Figure 4-5A). Neither L-NAME nor L-NPA had any effect on vascular response to E2 in males, suggesting that it is not NOS-dependent (Figure 4-5B).

4.3.5 Sex-Specific Differences in Vascular Protein Expression

There was no statistical difference in the total expression of either ERα or ERβ between female and male thoracic aortas (data shown in Appendix A-4). Total eNOS or nNOS content in the thoracic aortas was also not significantly different between sexes (Figure 4-6A and B). However, phosphorylation of nNOS is an important determinant of the enzyme activity. Interestingly, there was a greater baseline expression of both stimulatory (Ser1417) (1.9 ±0.2-fold) and inhibitory (Ser847) (1.7 ±0.3-fold) phosphorylated forms of nNOS in male compared to female vessels (Figure 4-6C and D). Unlike human endothelial phospho-nNOS, phospho-nNOS from the rat aortas demonstrated increased gel electrophoretic mobility. The latter depends on not only size, but also charge of proteins. Thus, although according to its amino acid composition the true

molecular weight of phospho-nNOS is higher, it reproducibly runs at a smaller apparent molecular weight. Such a discrepancy between actual and apparent molecular weights is not uncommon in western blotting applications.

4.3.6 nNOS, Unlike eNOS, Does Not Contribute to MCh-Induced Relaxation

An endothelium-dependent agonist, MCh, leads to VSMC relaxation via rapid release of endothelial vasoactive substances, including NO. We tested whether this MCh-stimulated NO is partially derived from nNOS. As illustrated by the Figure 4-6A, MCh sensitivity was significantly reduced in the presence of the general NOS inhibitor, L-NAME (MCh EC_{50}=0.28 ±0.09 μmol/l vs 0.04 ±0.01 μmol/l with and without L-NAME), but not the specific nNOS inhibitor, L-NPA (MCh EC_{50}=0.04 ±0.01 μmol/l with or without L-NPA). Similar responses to MCh were observed in both female (Figure 4-7A) and male (Figure 4-7B) mesenteric vascular beds. These results support the known role for NOS (inhibitable by L-NAME), likely to be eNOS, in the vasorelaxation elicited by stimulation of muscarinic receptors on the endothelium.

4.4 DISCUSSION

The key findings of the present study are following: (1) E2 rapidly increases nNOS activity and nNOS-mediated NO production in endothelial cells, (2) Endothelial-derived nNOS contributes to E2-induced vascular relaxation in

female but not male arteries, (3) There is a greater presence of nNOS in its phosphorylated state in male compared to female aortas.

The localization of nNOS in the vascular endothelium is a recent finding with the full implications yet to be understood. We validated basal expression of nNOS in human endothelial cells (HUVEC), as well as cross-sections of rat mesenteric arteries, where nNOS staining was strongly present in the endothelium and, to some extent, in the vessel media and adventitia. Historically, nNOS (named NOS I) was the first of NOS isozymes purified in 1990 from rat and porcine cerebellum,[21, 22] which today is known as a key source of NO in the nervous system. As a vital neurotransmitter system in brain development, defective NO/nNOS function has been implicated in devastating neurodegenerative processes such as dementia.[15] Other studies have linked nNOS hyperactivation to neuronal damage following cerebrovascular accident, i.e. stroke,[14, 23] suggesting a potential for nNOS inhibitors to ameliorate ischemic brain injury.[24] It is likely that understanding the role of vascular nNOS may as well uncover a far reaching significance in health and/or disease.

To our knowledge, the ability of E2 to rapidly activate nNOS in the endothelium has not been reported before. We found that E2 applied to endothelial cells in physiological concentrations increased levels of activating phospho1417-nNOS within 5 min, without affecting the levels of inhibitory phospho847-nNOS. Our observation complements two recent reports suggesting that E2 activates nNOS in neuronal cells[16] and VSMC[17] via the rapid change in its phosphorylation status. Moreover, the E2-stimulated nNOS activity in HUVEC

was ER-dependent and associated with an increased NO production sensitive to nNOS inhibition. Previously, E2-mediated phosphorylation and activation of eNOS in the vascular endothelium was thought to be the major signaling pathway underlying female-specific systemic vasoprotection. Of note, activating Ser1417 phospho-site of nNOS is deemed analogous to the established phosphorylation site of Akt at Ser1177 in eNOS.[25] On the other hand, Ser847 phospho-site is located within the autoinhibitory loop of nNOS and functions to stabilize the inactive enzyme conformation.[26] This may resemble the constituvely phosphorylated (inhibitory) Thr495 residue of resting eNOS, which is located within the CaM binding domain and stabilizes the inactive enzyme. Changes in Thr495 phosphorylation are generally associated with stimuli that elevate intracellular Ca^{2+} and increase eNOS activity many-fold (e.g., bradykinin and acetylcholine). However stimulation with estrogen does not appear to act on Thr495; rather it moderately increases NO production (2-4-fold over basal levels) via exclusively Ser1177 phosphorylation.[27]

It is important to note that many studies on the role of eNOS in vessel function were conducted using L-NAME, which is a non-selective NOS inhibitor (in fact, there are no selective eNOS inhibitors available to date). Thus, a cautious interpretation of results is warranted as we realize that eNOS is not the only NOS isoform in vascular cells, whereas some of the L-NAME effects may well be attributed to nNOS. Indeed, highly selective nNOS inhibition with 2 μmol/l L-NPA or the use of L-NAME, 100 μmol/l in the vessel bath experiments supported our hypothesis that E2-induced vascular relaxation was mediated, in

large part, by nNOS. The fact that both L-NPA and L-NAME had similar inhibitory effects on E2 relaxation suggests that nNOS appears to be a predominant signaling mechanism for E2. Although the possibility of eNOS contribution is not altogether excluded, there might be important differences in the functional role of these two NOS isoforms. For example, unlike eNOS, we and others have shown that nNOS expression extends beyond the endothelium, therefore endothelium-independent (i.e. VSMC) mechanisms can potentially contribute to vascular regulation. In our experimental protocols with endothelium-denuded arteries, the effect of nNOS inhibition was not evident until the higher doses of E2 (>10 μmol/l) were reached in the vessel bath (data shown in Appendix A-3). Although interpretation regarding the physiological role is difficult, this might be an indication of functional nNOS in VSMC. Likewise, it has been recently suggested that the VSMC-derived nNOS has a role in the relaxation of isolated porcine coronary arteries.[11] The identified mechanism for relaxation is via E2-initiated PI3K-Akt signaling, leading to rapid nNOS phosphorylation/activation and NO/cGMP-mediated opening of BK_{Ca} on VSMC. The authors propose this endothelium-independent mechanism to explain the clinical observation that E2 is able to enhance coronary blood flow in diseased coronary arteries with dysfunctional endothelium.[28]

Differences between sexes in vascular function are now well recognized at various levels, from epidemiological to the molecular.[29] Indeed, understanding the basis for these differences will likely lead to innovations in the CV medicine of near future. Relevant to our hypothesis, estrogen-eNOS signalosome constitutes

one of the important vasoprotective mechanisms under normal physiological conditions. Males (who are naturally deficient in this mechanism) demonstrate reduced compensatory reserve in NO-mediated vascular function in response to vascular risk factors.[30] Following this logic, numerous laboratory studies proved the concept that stimulation of NO-dependent mechanisms can as well benefit to male species treated with estrogen.[31, 32] Since a non-specific NOS inhibition was conducted in the above studies, the source of E2-enhanced NO production remained unclear. In the present study, we too found that E2 stimulation elicited lesser relaxation in male compared to female isolated vessels. There was also no effect of NOS or nNOS inhibition in male arteries, suggesting that unlike in females, E2 causes relaxation via other NOS-independent pathways. Since vascular expression of both classical ERs was not significantly different between females and males, potentially other factors (e.g., post-receptor events) might account for the sex differences in vasorelaxation to E2. Although nNOS expression in thoracic aorta was also not different between the sexes, we measured significantly greater presence of phospho-nNOS in male compared to female vascular tissue. To note, phosphorylated nNOS forms display multiple bands, which is not surprising given variations in the native protein itself. Indeed, vascular nNOS appear in multiple splice variants.[33] As a result, multiple nNOS bands in western blots have been observed by different groups,[34, 35] and may also reflect mixed endothelium- and VSMC-derived pools of nNOS. Speculatively, increased baseline phosphorylation of nNOS in males may indicate the pool of the enzyme that is not amenable to acute

regulation/phosphorylation by estrogen, although may contribute to the basal vessel tone. This may explain the observed sex differences in E2-mediated vascular responses.

Lastly, our data suggest that nNOS is not involved in the classical endothelium-dependent relaxation stimulated by MCh. In both male and female arteries, L-NAME resulted in a right shift of MCh CCRC, whereas L-NPA did not have a significant effect on MCh dose-response. These results are consistent with the previously known role for eNOS (rather than nNOS) in stimulated NO-dependent vasodilation to agonists (such as acetylcholine analogs) or shear stress. Indeed, there are speculations in the literature whether stimulated versus basal vasomotor tone might be regulated/subserved by different sources of NO, i.e. eNOS vs. nNOS, respectively.[9, 13] For example, some investigations showed a poor correlation between stimulated and basal NO-dependent vasodilation within the same vascular bed, where stimulated response tended to be lost in disease settings (termed “endothelial dysfunction”), while there was a relative preservation of the basal tonic NO generation.[36]

In summary, we demonstrated the presence of functional nNOS in the vascular cells. Stimulation of the vascular endothelium with physiological concentrations of E2 led to a rapid increase in activating phosphorylation of nNOS and nNOS-dependent NO production, a novel mechanism of estrogen action in this cell type. At the level of whole vessels *ex vivo* E2 elicited dose-dependent relaxation, largely via nNOS activation. Interestingly, this vascular mechanism was found in female but not male rat vessels, and was associated

with a greater presence of nNOS in a chronically phosphorylated state in males. Further research is needed to delineate the role and regulation of nNOS in vascular health and disease.

It is intriguing that now, two decades after nNOS was first described in neuronal tissue, nNOS inhibitors are being tested through different phases of clinical trials as potential therapeutic agents for neurological conditions. If better understood, vascular nNOS may as well yield yet unknown possibilities in vascular pharmacology.

4.5 REFERENCES

1. Orshal JM, Khalil RA. Gender, sex hormones, and vascular tone. *American journal of physiology.* 2004;286(2):R233-249.
2. Qiao X, McConnell KR, Khalil RA. Sex steroids and vascular responses in hypertension and aging. *Gender medicine.* 2008;5 Suppl A:S46-64.
3. Florian M, Lu Y, Angle M, Magder S. Estrogen induced changes in Akt-dependent activation of endothelial nitric oxide synthase and vasodilation. *Steroids.* 2004;69(10):637-645.
4. Haynes MP, Sinha D, Russell KS, Collinge M, Fulton D, Morales-Ruiz M, Sessa WC, Bender JR. Membrane estrogen receptor engagement activates endothelial nitric oxide synthase via the PI3-kinase-Akt pathway in human endothelial cells. *Circulation research.* 2000;87(8):677-682.
5. Chambliss KL, Shaul PW. Estrogen modulation of endothelial nitric oxide synthase. *Endocrine reviews.* 2002;23(5):665-686.
6. Haynes MP, Li L, Sinha D, Russell KS, Hisamoto K, Baron R, Collinge M, Sessa WC, Bender JR. Src kinase mediates phosphatidylinositol 3-kinase/Akt-dependent rapid endothelial nitric-oxide synthase activation by estrogen. *The Journal of biological chemistry.* 2003;278(4):2118-2123.

7. Bachetti T, Comini L, Curello S, Bastianon D, Palmieri M, Bresciani G, Callea F, Ferrari R. Co-expression and modulation of neuronal and endothelial nitric oxide synthase in human endothelial cells. *Journal of molecular and cellular cardiology.* 2004;37(5):939-945.

8. Daneshtalab N, Smeda JS. Alterations in the modulation of cerebrovascular tone and blood flow by nitric oxide synthases in SHRsp with stroke. *Cardiovascular research.* 2010;86(1):160-168.

9. Seddon MD, Chowienczyk PJ, Brett SE, Casadei B, Shah AM. Neuronal nitric oxide synthase regulates basal microvascular tone in humans in vivo. *Circulation.* 2008;117(15):1991-1996.

10. Capettini LS, Cortes SF, Gomes MA, Silva GA, Pesquero JL, Lopes MJ, Teixeira MM, Lemos VS. Neuronal nitric oxide synthase-derived hydrogen peroxide is a major endothelium-dependent relaxing factor. *Am J Physiol Heart Circ Physiol.* 2008;295(6):H2503-2511.

11. Han G, Ma H, Chintala R, Fulton DJ, Barman SA, White RE. Essential role of the 90-kilodalton heat shock protein in mediating nongenomic estrogen signaling in coronary artery smooth muscle. *The Journal of pharmacology and experimental therapeutics.* 2009;329(3):850-855.

12. Melikian N, Seddon MD, Casadei B, Chowienczyk PJ, Shah AM. Neuronal nitric oxide synthase and human vascular regulation. *Trends in cardiovascular medicine.* 2009;19(8):256-262.

13. Seddon M, Melikian N, Dworakowski R, Shabeeh H, Jiang B, Byrne J, Casadei B, Chowienczyk P, Shah AM. Effects of neuronal nitric oxide synthase on human coronary artery diameter and blood flow in vivo. *Circulation.* 2009;119(20):2656-2662.

14. Huang PL. Neuronal and endothelial nitric oxide synthase gene knockout mice. *Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas / Sociedade Brasileira de Biofisica ... [et al.* 1999;32(11):1353-1359.

15. Zhou L, Zhu DY. Neuronal nitric oxide synthase: structure, subcellular localization, regulation, and clinical implications. *Nitric Oxide.* 2009;20(4):223-230.

16. Gingerich S, Krukoff TL. Activation of ERbeta increases levels of phosphorylated nNOS and NO production through a Src/PI3K/Akt-

dependent pathway in hypothalamic neurons. *Neuropharmacology.* 2008;55(5):878-885.

17. Han G, Ma H, Chintala R, Miyake K, Fulton DJ, Barman SA, White RE. Nongenomic, endothelium-independent effects of estrogen on human coronary smooth muscle are mediated by type I (neuronal) NOS and PI3-kinase-Akt signaling. *Am J Physiol Heart Circ Physiol.* 2007;293(1):H314-321.

18. Chakrabarti S, Lekontseva O, Peters A, Davidge ST. 17beta-Estradiol induces protein S-nitrosylation in the endothelium. *Cardiovascular research.* 2010;85(4):796-805.

19. Chen Z, Yuhanna IS, Galcheva-Gargova Z, Karas RH, Mendelsohn ME, Shaul PW. Estrogen receptor alpha mediates the nongenomic activation of endothelial nitric oxide synthase by estrogen. *The Journal of clinical investigation.* 1999;103(3):401-406.

20. Parker RA, Huang Q, Tesfamariam B. Influence of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors on endothelial nitric oxide synthase and the formation of oxidants in the vasculature. *Atherosclerosis.* 2003;169(1):19-29.

21. Mayer B, John M, Bohme E. Purification of a Ca2+/calmodulin-dependent nitric oxide synthase from porcine cerebellum. Cofactor-role of tetrahydrobiopterin. *FEBS letters.* 1990;277(1-2):215-219.

22. Bredt DS, Snyder SH. Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proceedings of the National Academy of Sciences of the United States of America.* 1990;87(2):682-685.

23. Eliasson MJ, Huang Z, Ferrante RJ, Sasamata M, Molliver ME, Snyder SH, Moskowitz MA. Neuronal nitric oxide synthase activation and peroxynitrite formation in ischemic stroke linked to neural damage. *J Neurosci.* 1999;19(14):5910-5918.

24. Nanri K, Montecot C, Springhetti V, Seylaz J, Pinard E. The selective inhibitor of neuronal nitric oxide synthase, 7-nitroindazole, reduces the delayed neuronal damage due to forebrain ischemia in rats. *Stroke; a journal of cerebral circulation.* 1998;29(6):1248-1253; discussion 1253-1244.

25. Adak S, Santolini J, Tikunova S, Wang Q, Johnson JD, Stuehr DJ. Neuronal nitric-oxide synthase mutant (Ser-1412 --> Asp) demonstrates surprising connections between heme reduction, NO complex formation, and catalysis. *The Journal of biological chemistry.* 2001;276(2):1244-1252.

26. Rameau GA, Chiu LY, Ziff EB. Bidirectional regulation of neuronal nitric-oxide synthase phosphorylation at serine 847 by the N-methyl-D-aspartate receptor. *The Journal of biological chemistry.* 2004;279(14):14307-14314.

27. Fleming I, Busse R. Molecular mechanisms involved in the regulation of the endothelial nitric oxide synthase. *American journal of physiology.* 2003;284(1):R1-12.

28. Rosano GM, Sarrel PM, Poole-Wilson PA, Collins P. Beneficial effect of oestrogen on exercise-induced myocardial ischaemia in women with coronary artery disease. *Lancet.* 1993;342(8864):133-136.

29. Knot HJ, Lounsbury KM, Brayden JE, Nelson MT. Gender differences in coronary artery diameter reflect changes in both endothelial Ca2+ and ecNOS activity. *The American journal of physiology.* 1999;276(3 Pt 2):H961-969.

30. Kahonen M, Tolvanen JP, Sallinen K, Wu X, Porsti I. Influence of gender on control of arterial tone in experimental hypertension. *The American journal of physiology.* 1998;275(1 Pt 2):H15-22.

31. Sobey CG, Weiler JM, Boujaoude M, Woodman OL. Effect of short-term phytoestrogen treatment in male rats on nitric oxide-mediated responses of carotid and cerebral arteries: comparison with 17beta-estradiol. *The Journal of pharmacology and experimental therapeutics.* 2004;310(1):135-140.

32. Chan YC, Leung FP, Wong WT, Tian XY, Yung LM, Lau CW, Tsang SY, Yao X, Chen ZY, Huang Y. Therapeutically relevant concentrations of raloxifene dilate pressurized rat resistance arteries via calcium-dependent endothelial nitric oxide synthase activation. *Arteriosclerosis, thrombosis, and vascular biology.* 2010;30(5):992-999.

33. Wilcox JN, Subramanian RR, Sundell CL, Tracey WR, Pollock JS, Harrison DG, Marsden PA. Expression of multiple isoforms of nitric oxide

synthase in normal and atherosclerotic vessels. *Arteriosclerosis, thrombosis, and vascular biology.* 1997;17(11):2479-2488.

34. Chaudhury A, Rao YM, Goyal RK. PIN/LC8 is associated with cytosolic but not membrane-bound nNOS in the nitrergic varicosities of mice gut: implications for nitrergic neurotransmission. *Am J Physiol Gastrointest Liver Physiol.* 2008;295(3):G442-451.

35. Jin XG, He SQ, Yan XT, Zhang G, Wan L, Wang J, Li Y, Tian X, Tian Y, Luo A. Variants of neural nitric oxide synthase in the spinal cord of neuropathic rats and their effects on nuclear factor-kappaB (NF-kappaB) activity in PC12 cells. *J Pain.* 2009;10(1):80-89.

36. Deanfield JE, Halcox JP, Rabelink TJ. Endothelial function and dysfunction: testing and clinical relevance. *Circulation.* 2007;115(10):1285-1295.

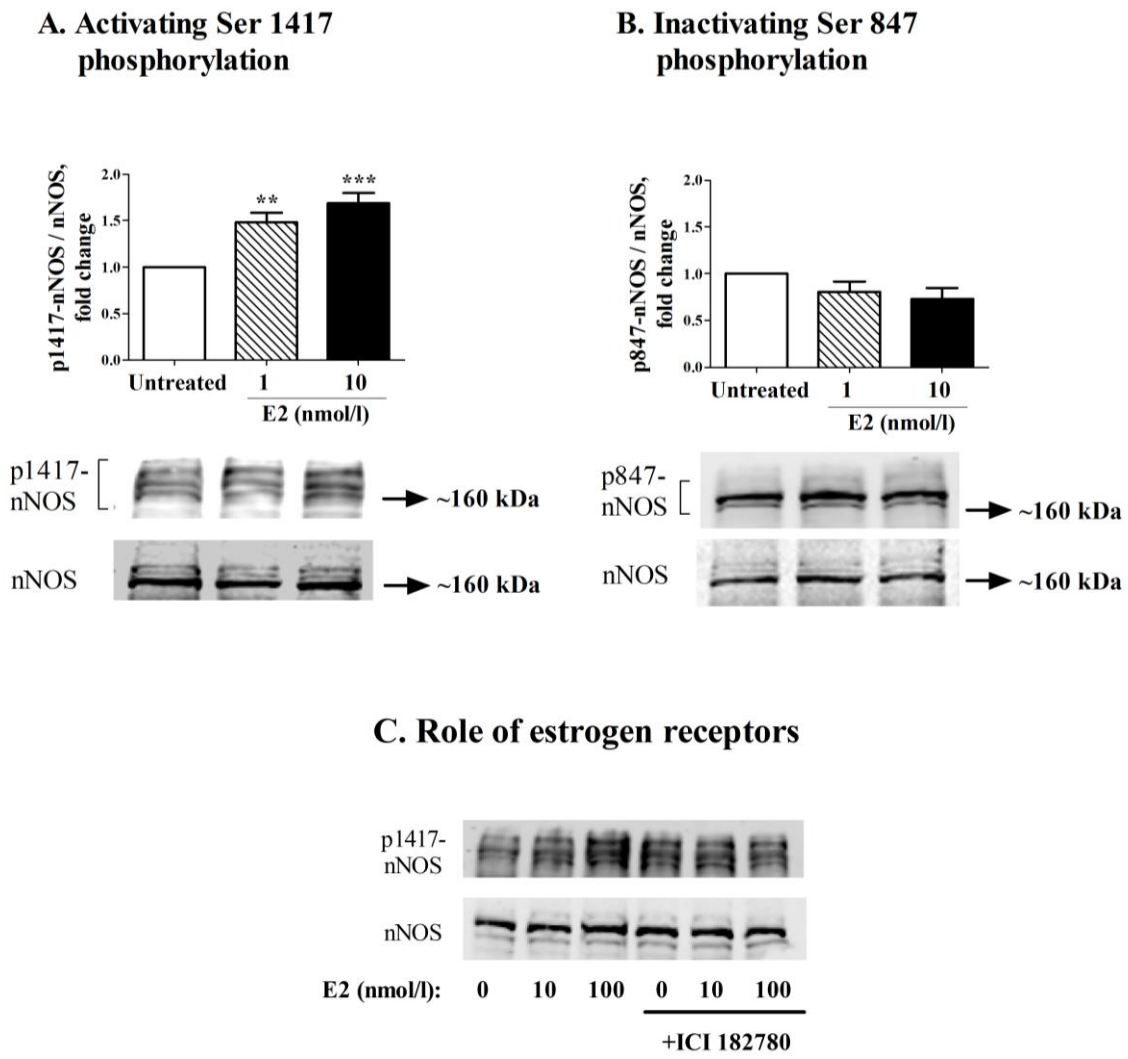

Figure 4-1. E2 acutely activates nNOS in HUVECs

Representative immunoblots from lysates of HUVECs stimulated with E2 for 5 min. The lysates were probed with antibodies against phosphoSer1417-nNOS (panel A and C) or phosphoSer847-nNOS (panel B). P-nNOS bands were normalized to the corresponding total nNOS. Data presented as mean ± SEM, n=6-7. ** $p<0.01$, *** $p<0.001$ compared to the untreated control. Panel C: HUVECs were pre-treated with ICI 182780 (10 μmol/l), the classical ER antagonist, for 1 hr prior to 5-min stimulation with E2. Representative images from 2 independent experiments are shown.

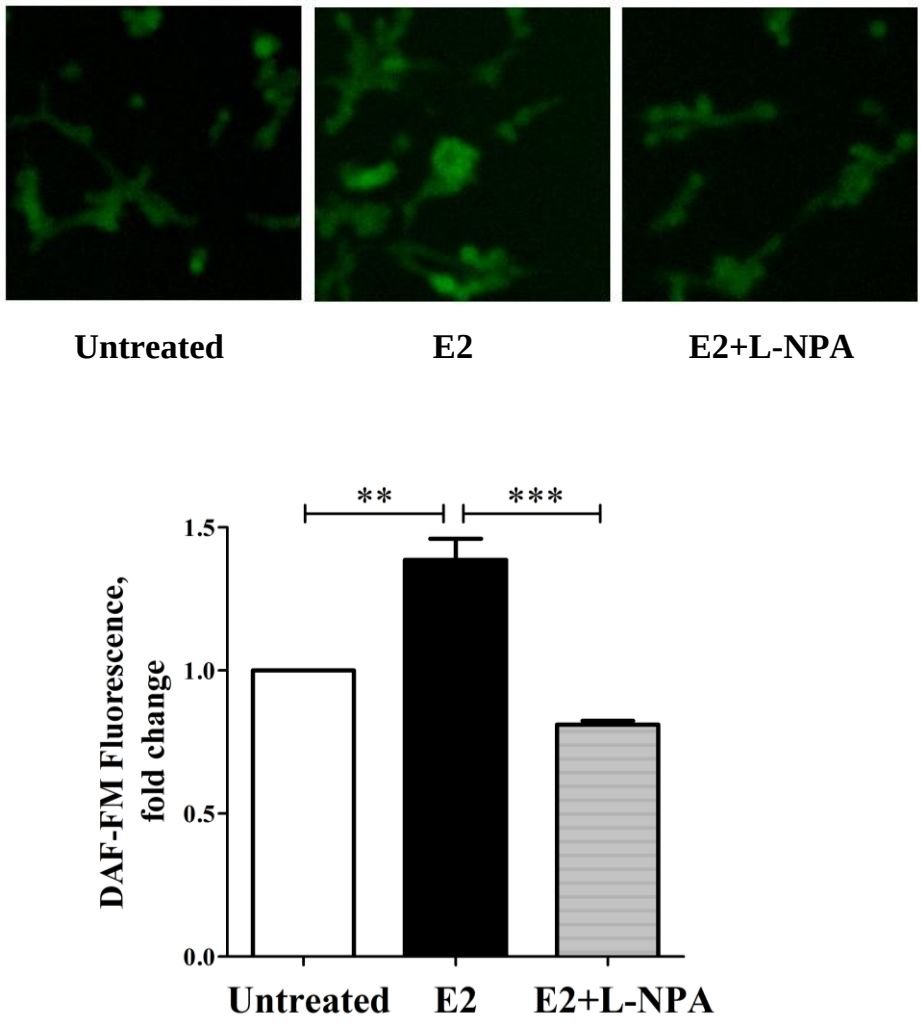

Figure 4-2. E2-stimulated NO production in HUVEC is nNOS-dependent

HUVECs were pre-treated with L-NPA (2 μmol/l) for 30 min prior to a 30-min stimulation with E2 (10 nmol/l). Cells were washed and treated with DAF-FM (10 μmol/l) for NO detection. Representative images are shown. Data presented as mean ± SEM of 3 independent experiments. ** $p<0.01$, *** $p<0.001$.

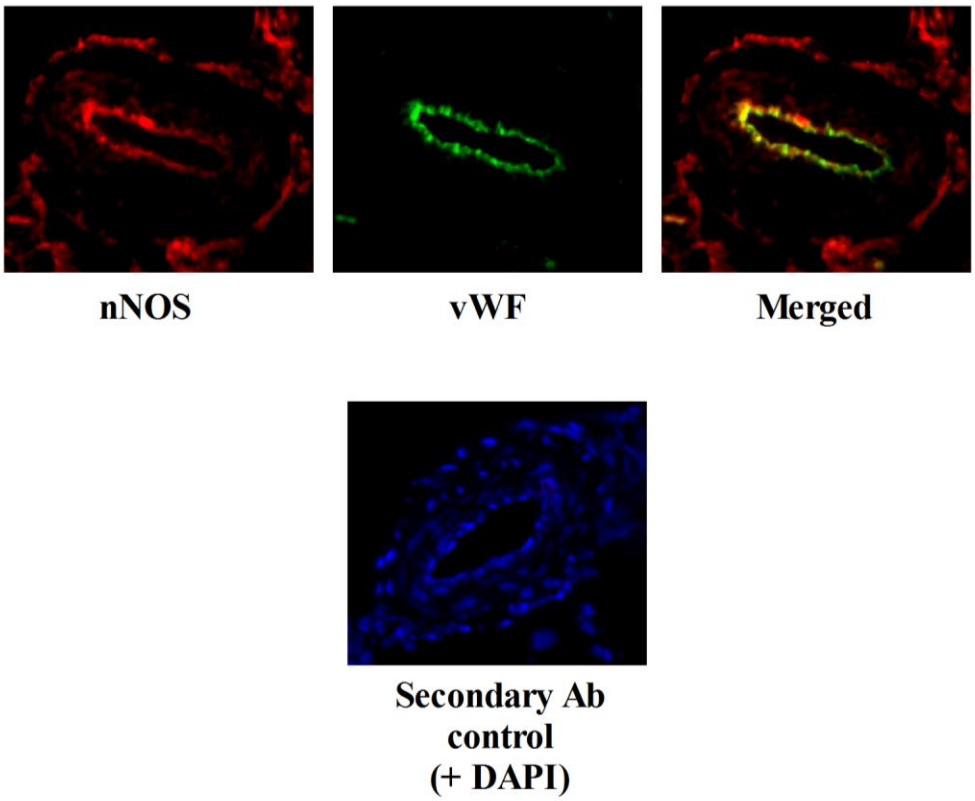

Figure 4-3. nNOS expression in the rat mesenteric artery

Cross-sections of mesenteric arteries from female Sprague Dawley rats were immunostained for nNOS and the endothelium-specific marker, von Willebrand's factor (vWF). A control image, from which the background autofluorescence was estimated and then subtracted, is shown with the vascular cells nuclei visualized by DAPI stain. A set of representative images from 3 independent experiments are shown.

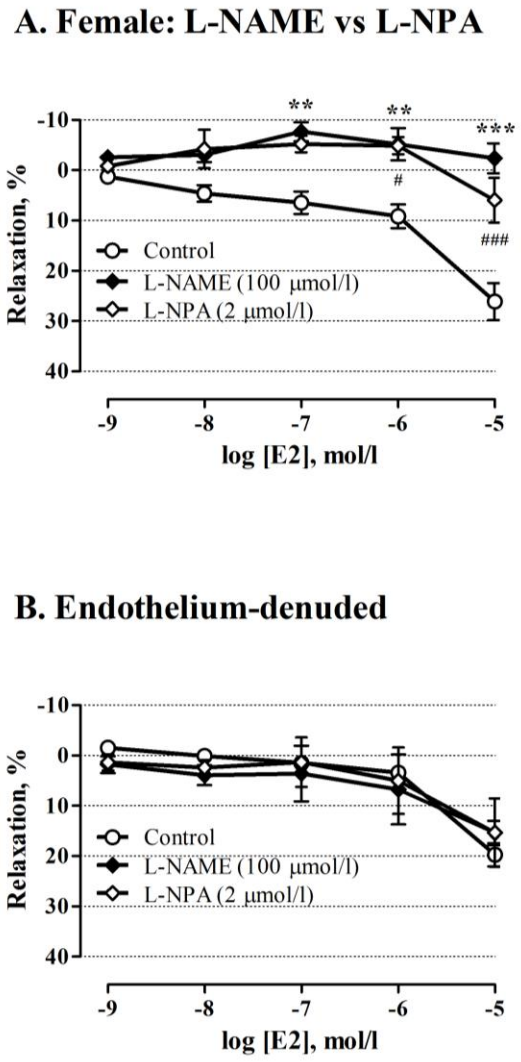

Figure 4-4. nNOS mediates acute E2-induced vascular relaxation in females

A: Dose-dependent relaxation to E2 was reduced in the presence of L-NAME (100 µmol/l) or L-NPA (2 µmol/l) in mesenteric arteries with intact endothelium. B: In endothelium-denuded vessels, NOS inhibition did not have an effect on response to E2. Data presented as means ± SEM, n=5-10. ** $p<0.01$, *** $p<0.001$ for L-NAME treated vessel vs control. $^{\#}$ $p<0.05$, $^{\#\#\#}$ $p<0.001$ for L-NPA treated vessel vs control.

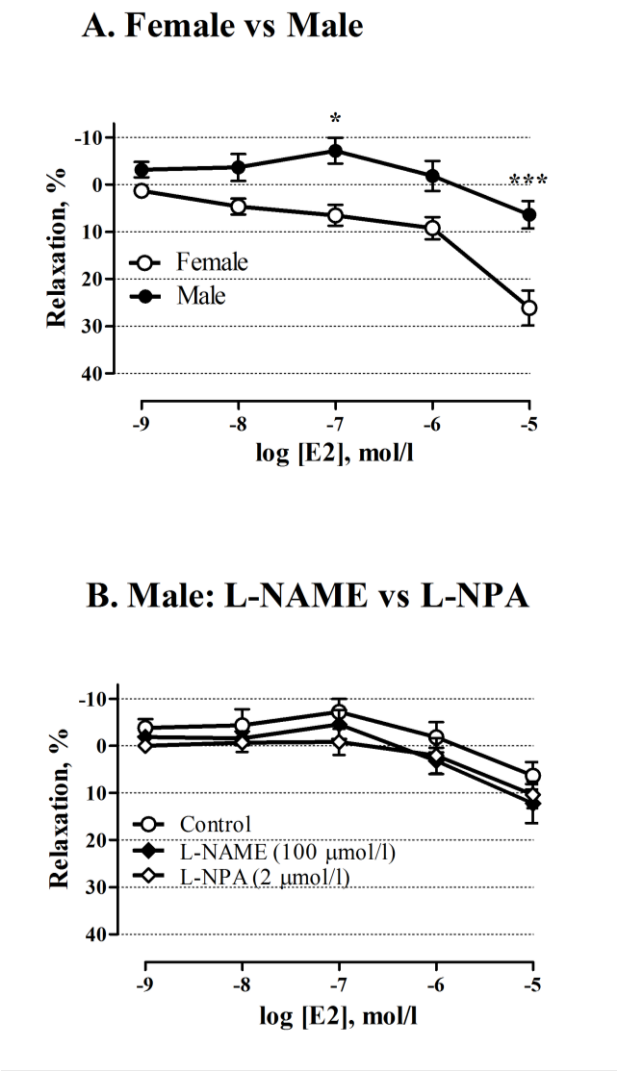

Figure 4-5. Vascular response to E2 in male rats does not depend on NOS

A: Dose-dependent relaxation of mesenteric arteries to E2 was reduced in male compared to female rats. B: In males, pre-incubation of vessels with either L-NAME (100 µmol/l) or L-NPA (2 µmol/l) for 30 min had no effect on E2 response. Data presented as means ± SEM, n=6-10. * $p<0.05$, *** $p<0.001$ male vs female (two-way ANOVA).

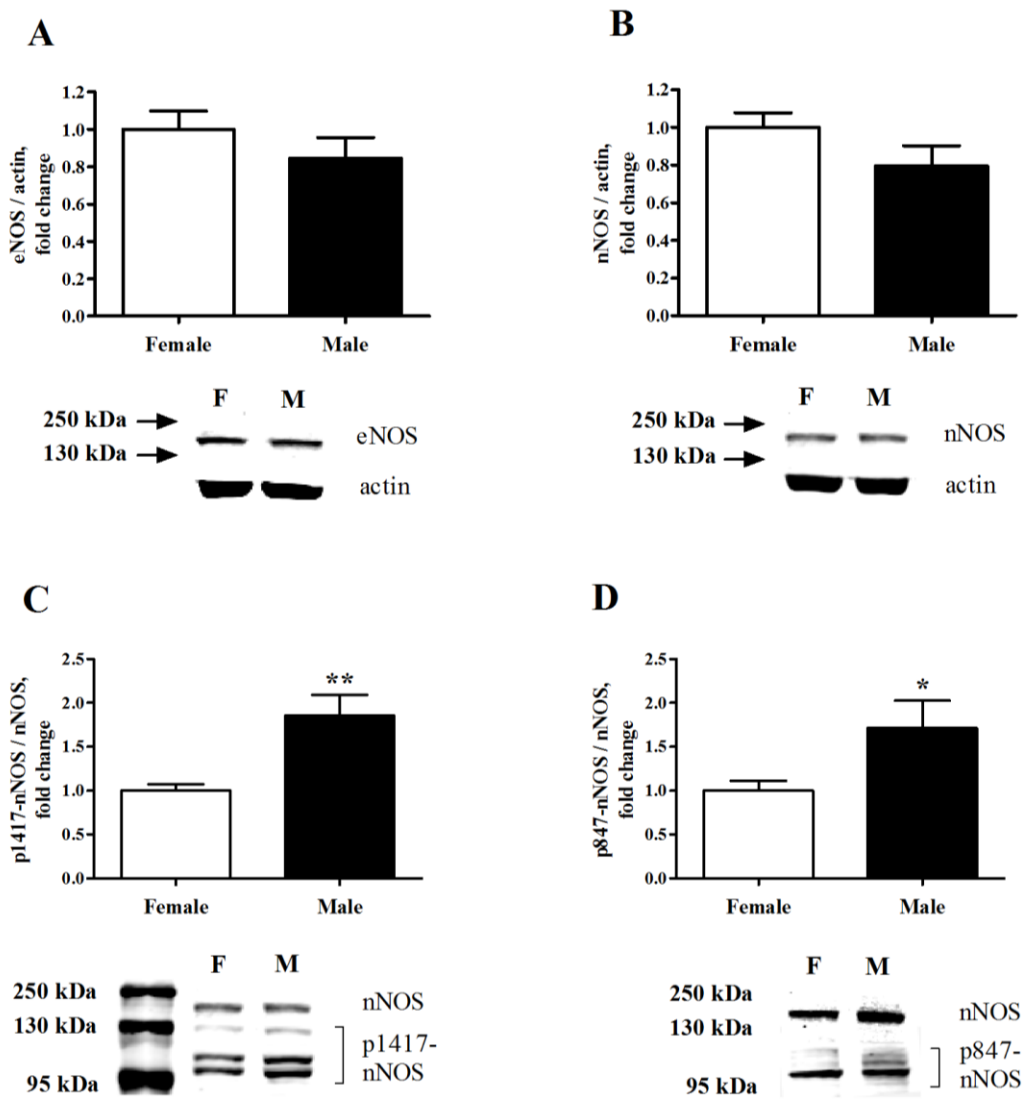

Figure 4-6. Greater presence of nNOS phosphorylation in males

Protein was extracted from rat thoracic aortas. Representative immunoblots are shown. The expression of total eNOS (A) and nNOS (B) was normalized to the corresponding actin bands. The two phosphorylated forms of nNOS (C and D) were normalized to the total nNOS. Male/female fold change was calculated and the results presented as means ± SEM, n=8 samples/group. * $p<0.05$, ** $p<0.01$.

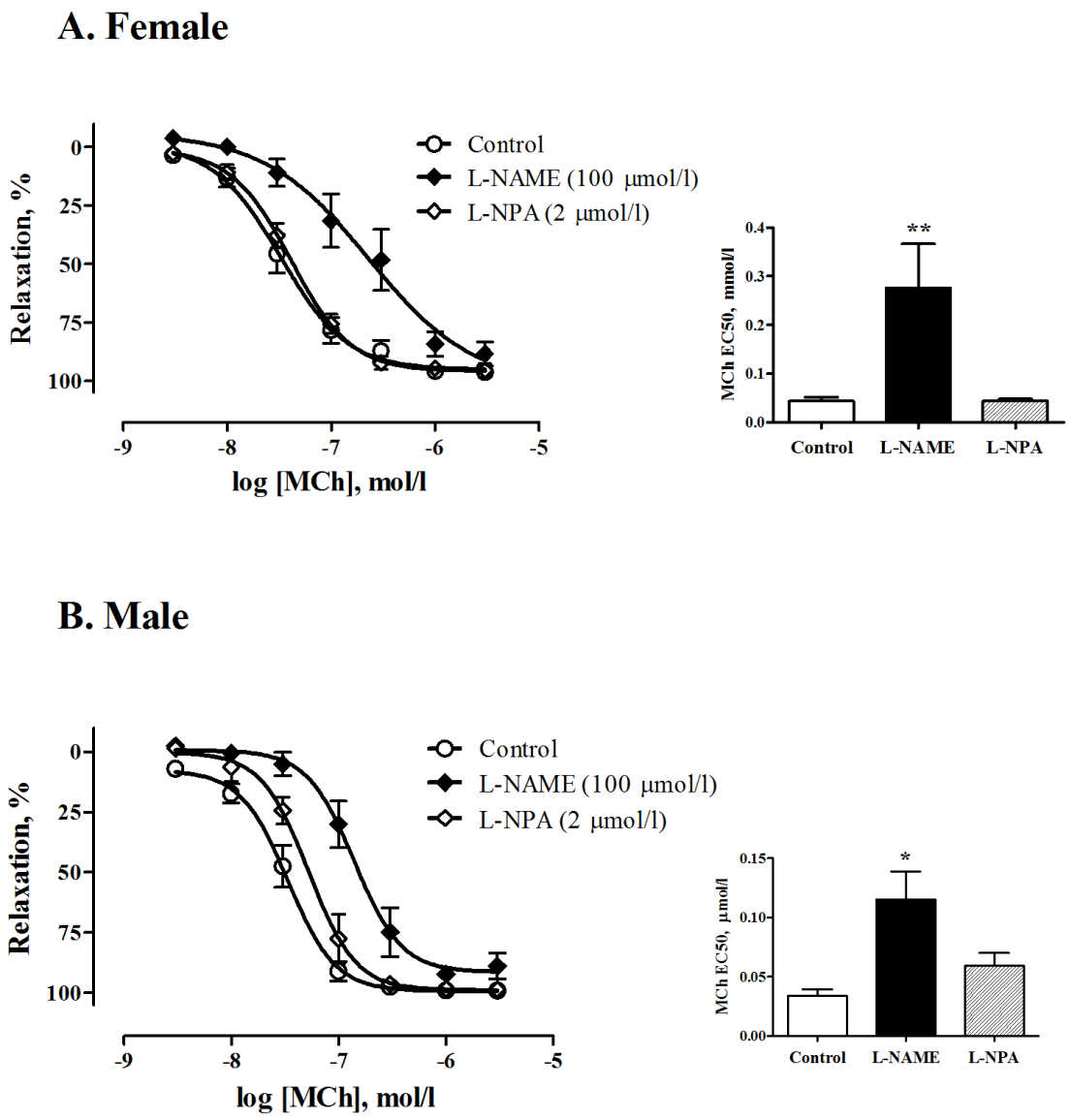

Figure 4-7. nNOS, unlike eNOS, does not have a role in MCh-induced relaxation

Either in female (A) or male (B) rats nNOS inhibition with L-NPA (2 µmol/l) did not have an effect on MCh-dependent relaxation in mesenteric arteries. In contrast, general NOS inhibitor, L-NAME (100 µmol/l) attenuated responses to MCh. Data presented as means ± SEM, n=6-10. * p<0.05, ** p<0.01.

CHAPTER 5:

ALTERED NEURONAL NITRIC OXIDE SYNTHASE IN THE AGING VASCULAR SYSTEM: IMPLICATIONS FOR ESTROGEN THERAPY

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Contribution: O Lekontseva (under the supervision of ST Davidge) designed the study, performed all the experiments (assisted by C Schleppe in DHE fluorescence) and data analyses, and prepared the first draft of the manuscript. Y Jiang performed vascular protein extraction and western blotting experiments. All authors participated in a critical review of the manuscript.

5.1 INTRODUCTION

Estrogen plays important systemic role in the regulation of blood pressure, vascular inflammation and oxidative balance, and is one of the factors contributing to a relatively favourable CV profile in females. Ovarian hormone deficiency resulting from either natural or surgical menopause, or ovarian dysfunction in premenopausal women increases the risk for CVD.[1-3] Although any hypoestrogenic state (regardless of causative or aging factor) correlates with the CVD risk, therapeutic effects of exogenous estrogen are age-dependent.[4-6] This has been referred to as the "timing hypothesis", which postulates that the vasoprotective potential of estrogen is lost in the aging microenvironment, presumably due to its altered molecular targets. However, the identity of these dysfunctional steps in the estrogen signaling has not been established.

Under physiological conditions, a spectrum of vasorelaxing, antiinflammatory and antioxidant actions of estrogen in the vascular system is NO dependent, and mediated through rapid calcium- and phosphorylation-dependent activation of eNOS.[7] A number of recent publications reported the constitutive presence of nNOS in vascular endothelial[8] and smooth muscle cells,[9] where it can, too, be rapidly activated on E2 exposure.[10, 11] Although the specific roles of vascular nNOS are not well explored, we demonstrated involvement of nNOS in E2-induced vascular relaxation in mesenteric arteries in female rats (Chapter 4).[11] Whether some of the natural antioxidant properties of estrogen are nNOS-dependent is not known.

NOS is a complex enzyme that relies on the availability of a number of cofactors and regulatory proteins to catalyze NO production (section 1.6). Some of these essential cofactors are known to be depleted in aging.[12, 13] In such an unfavorable biochemical environment, NOS is prone to uncoupling reaction resulting in the release of a highly reactive pro-oxidant, superoxide anion, with detrimental implications for vascular function. This idea has previously been worked up by White et al., who investigated role of nNOS in E2-induced relaxation in a model of endothelium-denuded porcine arteries. The study found that inhibition of the substrate binding to nNOS with synthetic L-arginine analogs (i.e. uncoupling) did not simply attenuate vascular relaxation to E2 but resulted in vasoconstriction, which was reversible with superoxide dismutase mimetic (Tempol) or superoxide scavenger (Tiron).[14] These findings suggest that E2 stimulation of uncoupled nNOS further potentiates superoxide generation. Thus, the same molecular mechanism of estrogen action, such as nNOS activation, may lead to dual outcomes depending on the vascular conditions.[15]

Given this background, we hypothesized that physiological function of nNOS is altered in the aging vascular system, where it is contributing to oxidative stress. We further hypothesized that E2 treatment has differential effects on vascular function in young versus aging conditions, in part due to the impaired nNOS-dependent mechanisms.

5.2 METHODS

5.2.1 Animal Model

Female Sprague Dawley rats 3 and 12 months old were purchased from Charles River Breeding Laboratories (Quebec, Canada) and housed in the animal facilities of the University of Alberta. The study was approved by the University of Alberta Animal Welfare Committee and conformed to the Canadian Council on Animal Care and the US NIH guidelines for the Care and Use of Laboratory Animals. Both young and aging rats were randomly assigned into one of the three groups: control (no intervention), Ovx placebo-treated or Ovx E2-treated. Bilateral ovariectomies were performed via a small midline incision under inhaled isoflurane anaestesia. At the time of surgery, rats were subcutaneously implanted with the placebo or the E2 pellet (0.5 mg/pellet, 60-day release, Innovative Research of America) for the duration of 4 weeks. The characteristics of this model were described in Chapters 2 and 3.[16, 17]

5.2.2 Vessel Isolation

Rats were euthanized by exsanguination under isoflurane anesthesia. Blood was collected from the chest cavity and serum prepared for subsequent measurements of E2 concentrations. Thoracic aortas were collected and stored at -80°C for further molecular studies. The upper portion of mesentery was excised and placed in ice-cold HEPES-buffered PSS (as described in Chapter 2). Mesenteric arteries (mean internal diameter 200 μm) were carefully dissected out from surrounding tissue and cut into 2 mm-long rings.

5.2.3 Vascular Function Protocol

Mesenteric artery function was assessed on a wire myograph system as described in Methods of Chapter 4. Briefly, after normalization to an optimal resting tension and an equilibration period, vessels were tested for smooth muscle and endothelial integrity with NA and MCh. Vascular responses to E2 (0.001-100 μmol/l) were recorded following a pre-constriction with the EC_{80} of NA. To assess the contribution of nNOS to E2-induced vasorelaxation, the selective (L-NPA, 2 μmol/l) or non-selective (L-NAME, 100 μmol/l) NOS inhibitor was applied to vessel bath for 30 min prior to preconstriction.

5.2.4 Western Blotting for Vascular nNOS Expression

Protein was extracted from rat thoracic aortas and its concentration in the sample was determined as described in Chapter 2. Impact of aging and ovariectomy on vascular nNOS expression was examined by western blotting. Protein sample, 18 μg/well, was loaded on 8% SDS polyacrylamide gels. Bands for nNOS (mouse monoclonal primary antibody from BD Biosciences 1/250 and Alexa Fluor 750 goat anti-mouse IgG from Invitrogen 1/20,000) were normalized to β-actin (rabbit polyclonal primary antibody from Abcam 1/2,000 and IRDye goat anti-rabbit IgG from LI-COR, 1/20,000). Images were obtained with Odyssey Infrared Imaging System (LI-COR Biosciences) and quantified by densitometry using the corresponding software, Odyssey V3.0. Data were expressed as fold change over the respective control samples run on the same gel.

5.2.5 Vascular Superoxide Detection

Thoracic aorta segments, embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek), were frozen in liquid nitrogen and stored at -80°C. 20 µm-thick cross-sections were prepared and glass slides were stored at -80°C. At the time of experiment, slides were allowed to thaw for 2 min, followed by three 2-min washes with room temperature Hank's Buffered Salt Solution (HBSS). After the washes, vessel specimens were incubated at 37°C in fresh HBSS with or without added NOS inhibitor, L-NAME (100 µmol/l), or selective nNOS inhibitor, L-NPA (2 µmol/l). Another selective nNOS inhibitor, N-(1-Imino-3-butenyl)-L-ornithine (L-VNIO, 2 µmol/l) has been used in some of the experiments to further confirm L-NPA dependent effects. Following a 30-min incubation period, HBSS was removed and dihydroethidium (DHE, 20 µmol/l) was added to each slide for another 30-min incubation at 37°C. After three 2-min washes, a small drop of HBSS was added over each vessel section and a glass cover slip was applied. Slides were examined immediately (in the order of their treatment) under the Olympus IX81 fluorescence microscope to visualize red fluorescent product formed upon reaction of DHE with superoxide anions. Images were obtained and normalized for intraexperimental fluorescence using SlideBook software. Mean DHE fluorescence intensity was quantified using Adobe Photoshop Elements 2.0.

5.2.6 Statistical Analysis

Data were graphed and analyzed using GraphPad Prism 5 software, and are shown as mean ± SEM. Percent vascular relaxation to E2 was calculated using the formula: (Tpreconstricted - Tx)/(Tpreconsricted - Tbaseline)*100, as described in Methods of Chapter 4. Resulting E2 relaxation curves were summarized as AUC, which were compared within the age group using one-way analysis of variance (ANOVA) followed by Bonferroni's test. nNOS expression was compared with *t*-test between the control young and aging rats, whereas the effect of interventions (Ovx ± E2) was evaluated separately for each age group using one-way ANOVA. Effect of general NOS or selective nNOS inhibition on superoxide production was analyzed in each of the six animal groups by Kruskal-Wallis test followed by Dunn's post test. A p value < 0.05 was accepted as statistically significant.

5.3 RESULTS

5.3.1 Serum E2 levels

Ovariectomy resulted in low circulating E2 levels in both young (35.3 ±2.0 pmol/l) and aging rats (31.2 ±1.2 pmol/l), which corresponded with the marked uterine atrophy (uterus/body wt = 0.40 ±0.03 g and 0.60 ±0.08 g in young and aging, respectively). Serum E2 levels in hormone-replaced animals were 283.6 ±109.3 pmol/l (young) and 253.3 ±86.2 pmol/l (aging), with the consequent throphic effect on the uteri (uterus/body wt = 2.0 ±0.2 g and 2.5 ±0.2 g in young

and aging, respectively). There was a cyclicity in E2 levels in young intact females (mean 70.8 ±15.0 pmol/l), and variable E2 levels in aging intact females (mean 58.0 ±11.9 pmol/l), where the uterus/body wt = 1.7 ±0.1 g and 1.6 ±0.1 g, respectively.

5.3.2 Differential Impact of Ovariectomy on E2-mediated Vascular Function in Young vs. Aging Rats

E2 caused dose-dependent relaxation of mesenteric arteries, which was similar between young and aging intact animals (Figure 5-1A and B, open circles). Ovx led to a significant attenuation of vascular response to E2 in young rats, which returned to normal in E2-treated group (Figure 5-1A). In contrast, Ovx in aging significantly increased vascular relaxation to E2, and this response did not fully normalize in rats treated with E2 (Figure 5-1B).

5.3.3 Loss of nNOS Modulation of E2 Relaxation in Ovx

In the young vascular system, pre-incubation of arteries with L-NAME or L-NPA significantly attenuated vasorelaxation to E2 as statistically demonstrated via reduction in AUC (Figure 5-2A). There was no significant difference between the E2 responses obtained in the presence of either general or selective nNOS inhibitor, suggesting that E2 relaxation was mediated largely via nNOS. In Ovx group, NOS inhibition had no further effect on already diminished vessel response to E2 (Figure 5-2B). Interestingly, E2 relaxation curves in Ovx state closely mimicked responses of the arteries from normal rats where NOS was

inhibited (Figure 5-2A vs. B). Although vascular responses in Ovx E2-treated rats were similar to control rats, there was no role for NOS in the acute E2 signaling in these animals (Figure 5-2C).

5.3.4 nNOS and E2 Relaxation in Aging

In intact aging arteries, E2-mediated relaxation was significantly inhibited by L-NAME only, but not L-NPA (Figure 5-3A). This suggests loss of nNOS modulation of E2 response in the normal aging process. Likewise, NOS did not play role in the E2 relaxation in aging Ovx animlas, treated with either placebo (Figure 5-3B) or E2 (Figure 5-3C).

5.3.5 Vascular Expression of nNOS in Aging and Ovx

Tissue levels of nNOS were compared in young and aging thoracic aortas. Baseline nNOS expression was not different between young and aging vasculature (Figure 5-4A). Ovariectomy with or without E2 replacement, regardless of age, did not have a statistically significant effect on constitutive vascular nNOS levels (Figure 5-4B and C).

5.3.6 nNOS and Vascular Superoxide Production in Aging and Ovx

Although long-term nNOS expression in the vascular tissue did not significantly change with aging, the possibility of the enzyme dysfunction/uncoupling has been previously reported. We therefore determined whether the uncoupling of nNOS accounted for the loss of its role in the rapid E2-

mediated vascular relaxation in aging and Ovx. Thus, we assessed the contribution of NOS to superoxide production in our model. In the young control rats, the *in situ* superoxide production as measured by DHE fluorescence was negligible, however it increased in the vessel cross-sections pre-treated with L-NPA (Figure 5-5A). This suggests that under normal conditions the nNOS function is, as expected, to suppress oxidative stress. In contrast, in aging and/or Ovx state, nNOS inhibition reduced superoxide (Figure 5-5B, D, and E), suggesting that nNOS itself was contributing to superoxide generation in altered vascular conditions. The results obtained using another selective nNOS inhibitor, L-VNIO, were similar to L-NPA. Interestingly, in E2-treated rats, either young or aging, the superoxide levels were not dependent on NOS as DHE signal was not affected by NOS inhibition (Figure 5-5C and F).

5.4 DISCUSSION

This study demonstrated that whereas dose-dependent vascular relaxation to E2 was not changed with aging, ovarian deficiency had differential impact on these acute *ex vivo* E2 responses in young versus aging arteries. Chronic E2 replacement normalized vascular function in young Ovx but not aging Ovx rats. nNOS contribution to E2 vasorelaxation (that was observed in control young rats) was not retained in aging or Ovx conditions, and was not restored following the long-term E2 treatment. Although total vascular nNOS expression did not change, functionally the enzyme became a source of superoxide in aging

and Ovx rats. Altogether, these findings suggest that dysfunction at the level of nNOS may explain inefficient estrogen signaling in the aging vascular system.

Using an example of a vasoconstrictor pathway in a similar rat model, we have shown that age-related vascular dysfunction in females is potentiated by ovarian hormone deprivation (Chapter 2).[16] However, little is understood to what extent the protective effects of estrogen under standard physiological conditions can function in the aging state. A few previous studies have demonstrated attenuation of vascular protection in E2 supplementation experiments in aging rodents and late postmenopausal women. For example, in the study by Miller at al., carotid artery response to ballon injury was measured in young Ovx and aging Ovx rats that were treated with either placebo or E2, comparable to our model. Whereas E2 markedly attenuated neointima formation and expression of pro-inflammatory mediators in injured arteries from young rats (compared to placebo group), there was no reduction in inflammatory response seen in the aging E2-treated animals.[18] To a similar end, Sherwood et al. revealed favorable effects of short-term transdermal E2 on brachial artery flow-mediated dilation in women aged 50-59, but not 60-69 or 70-79 years old, in a crossover study.[19]

The mechanisms underlying the loss (or reversal) of vasoprotective potential of estrogen in aging are not well understood. Under physiological conditions, NO is an important mediator of rapid E2 signaling leading to vascular relaxation. Interestingly, we have recently found that E2-mediated vasoactivity in female rat resistance arteries appears largely dependent on nNOS isozyme present in the endothelium (potentially, also smooth muscle) (described in

Chapter 4). Indeed, both general NOS inhibitor (L-NAME) and highly selective nNOS inhibitor (L-NPA) attenuated E2 response to a comparable extent.[11] We now observed that in normal physiological aging, this role for nNOS in the acute vasorelaxation elicited by E2 was no longer present, since only L-NAME (most likely, inhibiting eNOS) but not L-NPA had the capacity to reduce E2 dose-response. Furthermore, ovariectomy in either young or aging rats resulted in the complete loss of NOS-dependent (including nNOS) component in E2 relaxation. This agrees with other reports in the literature. In particular, LeBlanc et al. also demonstrated lack of NOS modulation of coronary artery function (assessed by flow-mediated dilation) after ovariectomy in rats of different ages.[20] However, since only L-NAME was used in the latter study, the specific roles of eNOS versus nNOS remained unknown. It is yet becoming important to differentiate between these two constitutive enzymes in vascular cells as they appear to mediate distinct regulatory pathways in vascular health and disease.[21] In our model, nNOS-mediated relaxation affected by Ovx was not reestablished in animals chronically treated with E2. This observation suggests that other ovarian hormones or other mechanisms might be involved in nNOS regulation. For example, a series of recent reports indicated the importance of intact ovarian cycle with the natural hormonal fluctuations for nitrergic system in the brain. In this work examining mating behavior of female rats, both estrogen and progesterone were found necessary and synergistic in regulating nNOS and NO-mediated neuronal function in certain regions of the brain regulating the corresponding behavior.[22, 23]

Despite the altered vasoactive role, we found no significant change in total nNOS expression in vascular tissue with age or Ovx. Another study on a similar rat model reported that eNOS and iNOS expression in thoracic aortas also did not differ among the animals regardless of aging or Ovx status, although surprisingly nNOS detection was not aimed.[24] Whereas plasma levels of NO metabolites, nitrite and nitrate, remained also unchanged with age and/or Ovx, the downstream signaling mechanisms (including expression of sGC, etc.) were impaired in aging Ovx rats but not younger Ovx counterparts.[24] Our findings suggest a similar idea of underlying dysfunction at the level of nNOS (or downstream) despite its stable tissue expression. In contrast, other investigators clearly demonstrated reduced nNOS expression in Ovx rats in association with increased sympathetic vasoconstriction in hindlimb.[25] In this work, the levels of nNOS in skeletal muscle homogenates highly correlated with plasma E2 (but not progesterone) concentrations. Although animals of only young age were chosen as a study model, E2 replacement in Ovx rats fully restored nNOS expression and NOS modulation of sympathetic vascular tone in contracting muscle.[25] Such an upregulation of nNOS resulting from long-term E2 exposure likely suggests a genomic mechanism of estrogen action that has led to an apparently favorable outcome for the vascular regulation, at least in the young background.

In our study, despite similar aortic nNOS expression in all the animal groups, there was a dramatic shift of its enzymatic function towards superoxide production with aging or Ovx. An interesting aspect of our findings is that although chronic E2 treatment did not restore rapid nNOS-mediated vascular

relaxation, it appeared to improve coupling of this enzyme in both young and aging vessels. Indeed, no effect of NOS inhibition on vascular superoxide production was seen in E2-replaced animals, suggesting other sources of this radical. In contrast, in placebo-treated Ovx rats, NOS inhibition significantly lowered superoxide levels. A number of mechanisms were found to be involved in NOS uncoupling, with most consistent evidence implicating deficiency of essential cofactors such as BH_4, or the substrate L-arginine.[26] Uncoupled reaction is detrimental to vascular function in many ways: superoxide acts as an NO scavenger, as well as a potent pro-oxidant that damages bioactive molecules and generates further reactive oxygen and nitrogen species. Oxidative stress itself perpetuates NOS uncoupling via mechanisms involving oxidation of BH_4, disrupted enzyme dimerization, and etc.[27] On the other hand, estrogen is known to improve NOS function (what has been shown, at least, for eNOS) via increasing endothelial bioavailability of BH_4,[28] or reducing endothelial production of ADMA, an endogenous competitor of substrate binding[29] – to name a few examples that may support our findings. Not surprisingly, physiological antioxidant properties of nNOS (based on DHE experiments) were evident in young rats with intact ovarian function, which corresponded with nNOS-mediated vascular relaxation observed in these control conditions only.

In summary, our data indicate that vascular impact of ovarian deficiency is age-dependent. E2 replacement normalized altered relaxation in young but not aging vascular system, suggesting that the hormonal effects are also age-dependent. Vascular nNOS, an important mediator of acute E2-induced vascular

relaxation in young cycling females, was not involved in this aspect of vessel tone regulation in aging or Ovx animals (regardless of treatment). Although, the expression of nNOS in the whole vessel homogenates did not change, its enzymatic function was impaired in aging and Ovx conditions, where it became a source of superoxide. Whereas chronic E2 treatment appeared effective in reducing nNOS-dependent oxidative stress, ultimately it did not lead to restoration of nNOS-mediated vascular relaxation. Taken together, the study suggest that dysfunction of nNOS-dependent mechanisms may underlie unequal therapeutic efficacy of estrogen in young versus aging vascular system.

5.5 REFERENCES

1. Kallen AN, Pal L. Cardiovascular disease and ovarian function. *Current opinion in obstetrics & gynecology.* 2011;23(4):258-267.

2. Christakou CD, Diamanti-Kandarakis E. Role of androgen excess on metabolic aberrations and cardiovascular risk in women with polycystic ovary syndrome. *Women's health (London, England).* 2008;4(6):583-594.

3. Yanes LL, Romero DG, Moulana M, Lima R, Davis DD, Zhang H, Lockhart R, Racusen LC, Reckelhoff JF. Cardiovascular-renal and metabolic characterization of a rat model of polycystic ovary syndrome. *Gender medicine.* 2011;8(2):103-115.

4. Naka KK, Kalantaridou SN, Bechlioulis A, Kravariti M, Kazakos N, Katsouras CS, Tsatsoulis A, Michalis LK. Effect of ethinylestradiol/cyproterone acetate on endothelial function in young non-obese women with polycystic ovary syndrome: a pilot study. *Gynecol Endocrinol.* 2011;27(9):615-621.

5. Harman SM, Vittinghoff E, Brinton EA, Budoff MJ, Cedars MI, Lobo RA, Merriam GR, Miller VM, Naftolin F, Pal L, Santoro N, Taylor HS, Black DM. Timing and duration of menopausal hormone treatment may affect

cardiovascular outcomes. *The American journal of medicine.* 2011;124(3):199-205.

6. Kodaman PH. Early menopause: primary ovarian insufficiency and surgical menopause. *Seminars in reproductive medicine.* 2010;28(5):360-369.

7. Zhu W, Smart EJ. Caveolae, estrogen and nitric oxide. *Trends in endocrinology and metabolism: TEM.* 2003;14(3):114-117.

8. Bachetti T, Comini L, Curello S, Bastianon D, Palmieri M, Bresciani G, Callea F, Ferrari R. Co-expression and modulation of neuronal and endothelial nitric oxide synthase in human endothelial cells. *Journal of molecular and cellular cardiology.* 2004;37(5):939-945.

9. Brophy CM, Knoepp L, Xin J, Pollock JS. Functional expression of NOS 1 in vascular smooth muscle. *American journal of physiology.* 2000;278(3):H991-997.

10. Han G, Ma H, Chintala R, Miyake K, Fulton DJ, Barman SA, White RE. Nongenomic, endothelium-independent effects of estrogen on human coronary smooth muscle are mediated by type I (neuronal) NOS and PI3-kinase-Akt signaling. *American journal of physiology.* 2007;293(1):H314-321.

11. Lekontseva O, Chakrabarti S, Jiang Y, Cheung CC, Davidge ST. Role of Neuronal Nitric Oxide Synthase in Estrogen-Induced Relaxation in Rat Resistance Arteries. *The Journal of pharmacology and experimental therapeutics.* 2011.

12. Santhanam L, Christianson DW, Nyhan D, Berkowitz DE. Arginase and vascular aging. *J Appl Physiol.* 2008;105(5):1632-1642.

13. Delp MD, Behnke BJ, Spier SA, Wu G, Muller-Delp JM. Ageing diminishes endothelium-dependent vasodilatation and tetrahydrobiopterin content in rat skeletal muscle arterioles. *The Journal of physiology.* 2008;586(4):1161-1168.

14. White RE, Han G, Dimitropoulou C, Zhu S, Miyake K, Fulton D, Dave S, Barman SA. Estrogen-induced contraction of coronary arteries is mediated by superoxide generated in vascular smooth muscle. *American journal of physiology.* 2005;289(4):H1468-1475.

15. White RE, Gerrity R, Barman SA, Han G. Estrogen and oxidative stress: A novel mechanism that may increase the risk for cardiovascular disease in women. *Steroids.* 2010;75(11):788-793.

16. Lekontseva ON, Rueda-Clausen CF, Morton JS, Davidge ST. Ovariectomy in aged versus young rats augments matrix metalloproteinase-mediated vasoconstriction in mesenteric arteries. *Menopause (New York, N.Y.* 2010;17(3):516-523.

17. Lekontseva O, Jiang Y, Davidge ST. Estrogen replacement increases matrix metalloproteinase contribution to vasoconstriction in a rat model of menopause. *Journal of hypertension.* 2009;27(8):1602-1608.

18. Miller AP, Xing D, Feng W, Fintel M, Chen YF, Oparil S. Aged rats lose vasoprotective and anti-inflammatory actions of estrogen in injured arteries. *Menopause (New York, N.Y.* 2007;14(2):251-260.

19. Sherwood A, Bower JK, McFetridge-Durdle J, Blumenthal JA, Newby LK, Hinderliter AL. Age moderates the short-term effects of transdermal 17beta-estradiol on endothelium-dependent vascular function in postmenopausal women. *Arteriosclerosis, thrombosis, and vascular biology.* 2007;27(8):1782-1787.

20. LeBlanc AJ, Reyes R, Kang LS, Dailey RA, Stallone JN, Moningka NC, Muller-Delp JM. Estrogen replacement restores flow-induced vasodilation in coronary arterioles of aged and ovariectomized rats. *Am J Physiol Regul Integr Comp Physiol.* 2009;297(6):R1713-1723.

21. Melikian N, Seddon MD, Casadei B, Chowienczyk PJ, Shah AM. Neuronal nitric oxide synthase and human vascular regulation. *Trends in cardiovascular medicine.* 2009;19(8):256-262.

22. Martini M, Pradotto M, Panzica G. Synergic effects of estradiol and progesterone on regulation of the hypothalamic neuronal nitric oxide synthase expression in ovariectomized mice. *Brain research.* 2011;1404:1-9.

23. Sica M, Martini M, Viglietti-Panzica C, Panzica G. Estrous cycle influences the expression of neuronal nitric oxide synthase in the hypothalamus and limbic system of female mice. *BMC neuroscience.* 2009;10:78.

24. Stice JP, Eiserich JP, Knowlton AA. Role of aging versus the loss of estrogens in the reduction in vascular function in female rats. *Endocrinology.* 2009;150(1):212-219.

25. Fadel PJ, Zhao W, Thomas GD. Impaired vasomodulation is associated with reduced neuronal nitric oxide synthase in skeletal muscle of ovariectomized rats. *The Journal of physiology.* 2003;549(Pt 1):243-253.

26. Andrew PJ, Mayer B. Enzymatic function of nitric oxide synthases. *Cardiovascular research.* 1999;43(3):521-531.

27. Sun J, Druhan LJ, Zweier JL. Dose dependent effects of reactive oxygen and nitrogen species on the function of neuronal nitric oxide synthase. *Archives of biochemistry and biophysics.* 2008;471(2):126-133.

28. Lam KK, Lee YM, Hsiao G, Chen SY, Yen MH. Estrogen therapy replenishes vascular tetrahydrobiopterin and reduces oxidative stress in ovariectomized rats. *Menopause (New York, N.Y.* 2006;13(2):294-302.

29. Monsalve E, Oviedo PJ, Garcia-Perez MA, Tarin JJ, Cano A, Hermenegildo C. Estradiol counteracts oxidized LDL-induced asymmetric dimethylarginine production by cultured human endothelial cells. *Cardiovascular research.* 2007;73(1):66-72.

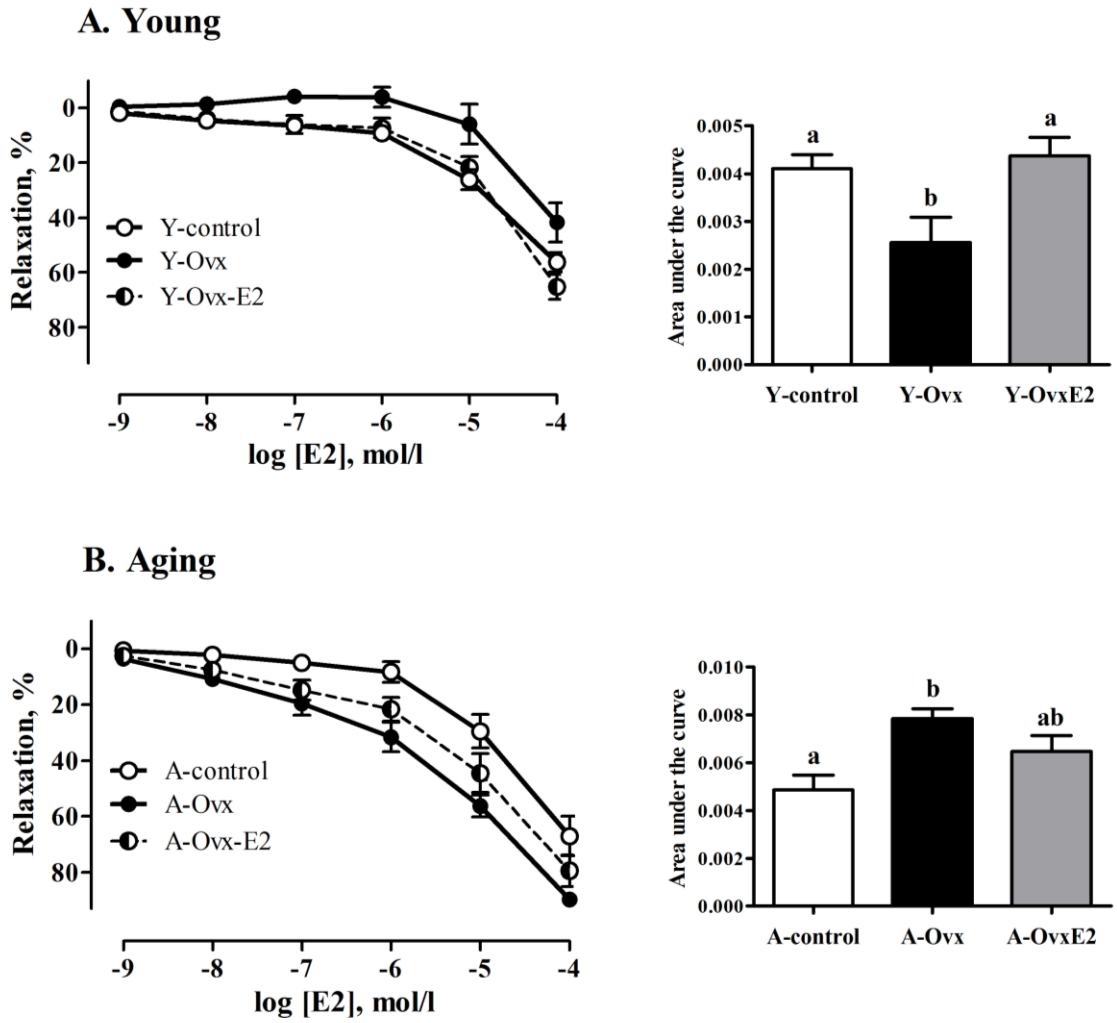

Figure 5-1. Impact of ovariectomy on vasorelaxation in young vs. aging

Mesenteric arteries were isolated from young or aging rats who were either intact or Ovx, treated with placebo or E2 for 4 weeks. Panel A: In young animals, dose-dependent relaxation to acute E2 was reduced by Ovx, but restored with chronic E2 treatment. Panel B: In aging animals, E2 response was increased in Ovx state, and did not fully normalize in E2-treated rats. Data are shown as mean ± SEM, n=10 animals/group. Symbols a and b indicate significant difference between the AUCs.

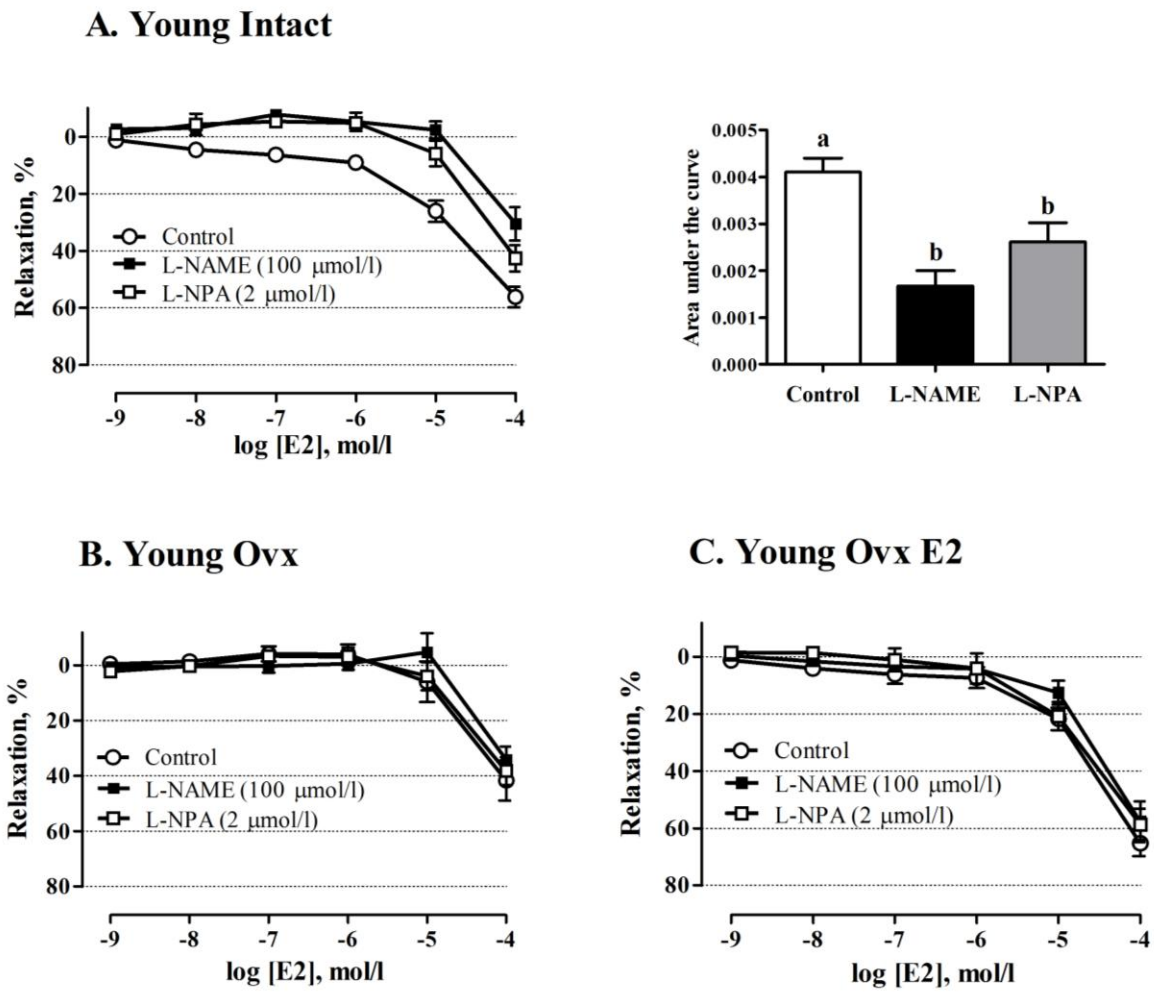

Figure 5-2. nNOS modulation of E2 relaxation in young vasculature

Dose-dependent relaxation to E2 was reduced in the presence of L-NAME (100 µmol/l) or L-NPA (2 µmol/l) in the arteries from young intact rats (Panel A). NOS inhibition had no effect on E2 responses in Ovx rats (Panel B), including those treated with E2 (Panel C). Data are shown as mean ± SEM, n=10. Symbols a and b indicate significant difference between the AUCs.

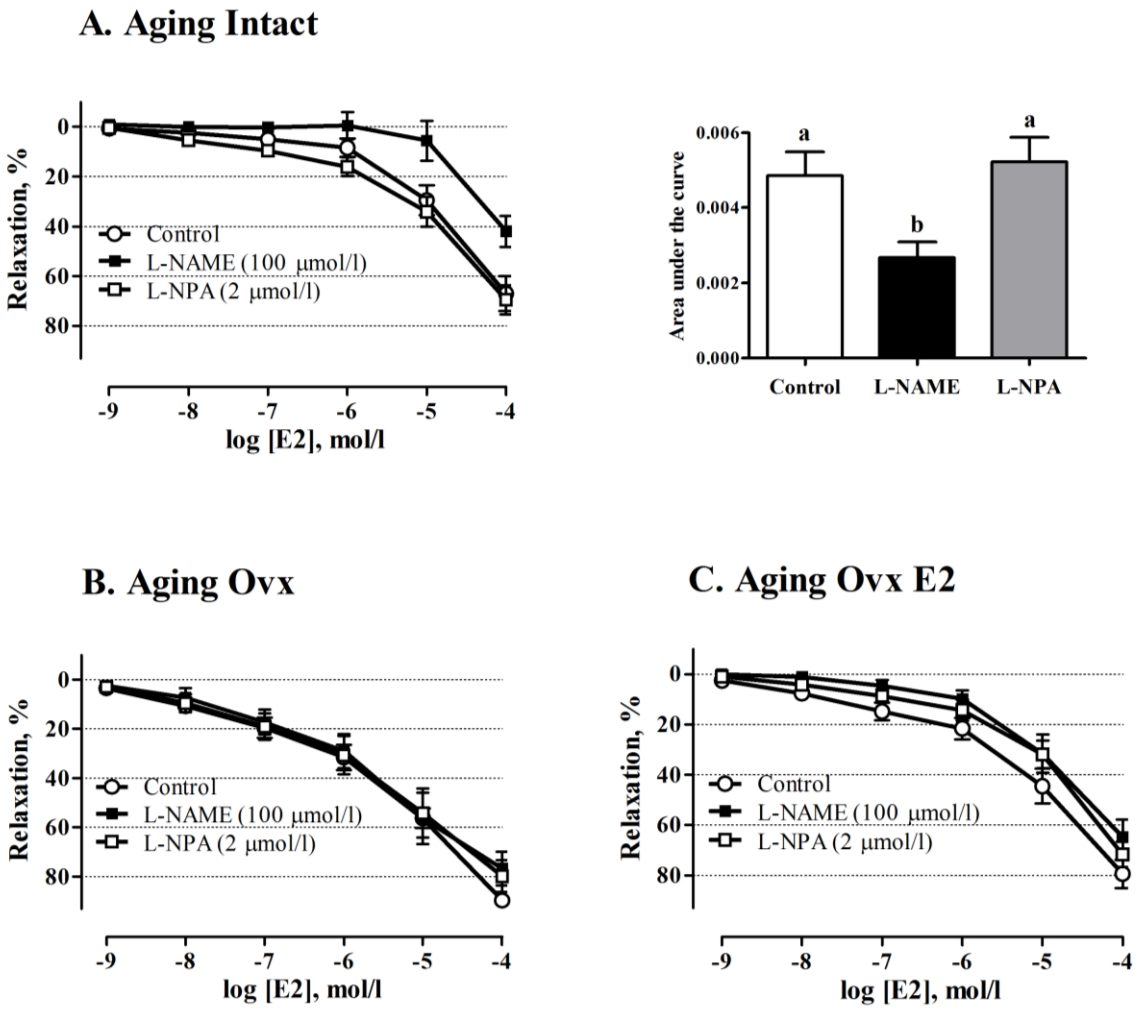

Figure 5-3. nNOS and E2 relaxation in aging vasculature

Dose-dependent relaxation to E2 was reduced in the presence of L-NAME (100 µmol/l), but not L-NPA (2 µmol/l) in the arteries from aging intact rats (Panel A). Neither L-NAME, nor L-NPA had an effect on E2 relaxation in Ovx rats, either placebo- or E2-treated (Panel B and C). Data are shown as mean ± SEM, n=10. Symbols a and b indicate significant difference between the AUCs.

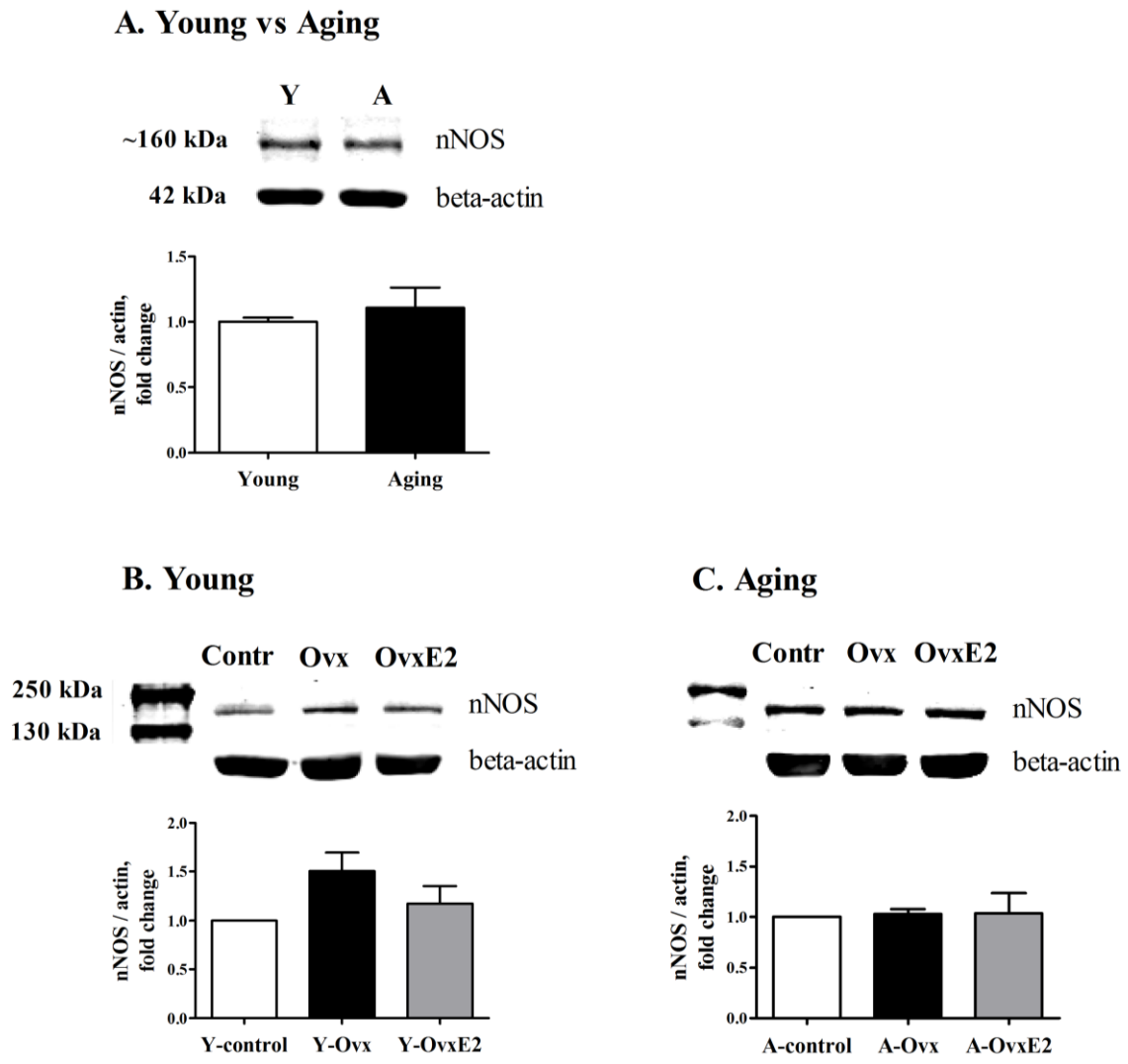

Figure 5-4. Vascular expression of nNOS: effect of aging and Ovx

Protein was extracted from rat thoracic aortas. Representative immunoblots are shown. The expression of total nNOS was normalized to the corresponding beta-actin bands and presented as fold change over the respective control. Data are shown as mean ± SEM of 4-6 experiments, *p*=ns.

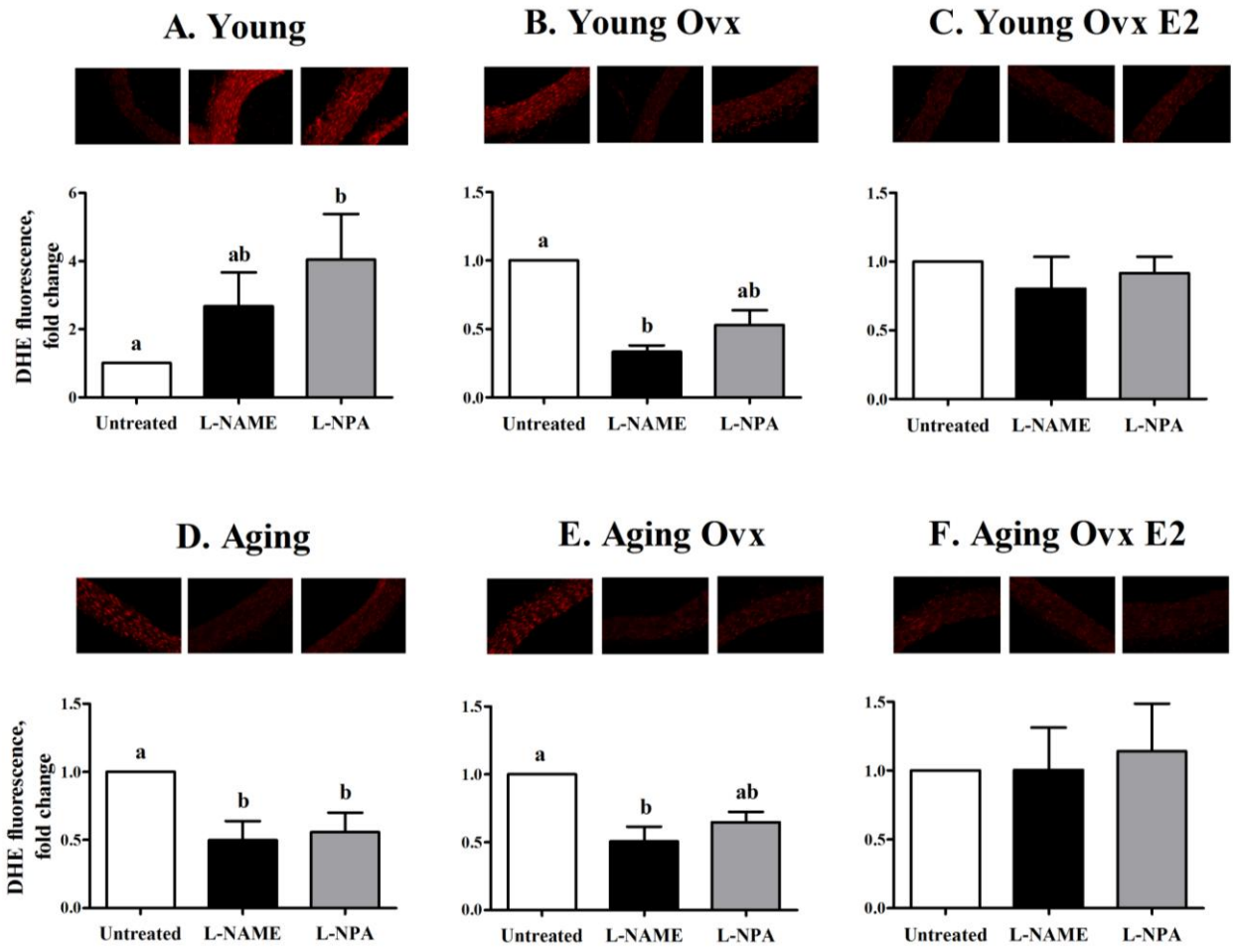

Figure 5-5. nNOS and vascular superoxide production in aging and Ovx*

Superoxide levels were measured by DHE fluorescence in the cross-sections of rat thoracic aortas. Effects of pan-NOS (L-NAME, 100 μmol/l) or selective nNOS inhibition (L-NPA, 2 μmol/l) on superoxide generation were assessed as a change in mean DHE fluorescence over the untreated control (DHE without NOS inhibitors). Impact of Ovx and E2 treatment on NOS function was examined in young (Panels A, B, C) and aging (Panels D, E, F) rats. Representative images are shown. The results from 5 independent experiments were summarized as mean ± SEM. Symbols a and b indicate significant difference.

* *Comparative baseline superoxide levels (i.e. untreated vessels) in the animal groups are shown in Appendix A-4.*

CHAPTER 6:

GENERAL DISCUSSION

6.1 SUMMARY

Aging is a complex process associated with systemic vascular dysfunction years preceding a clinical diagnosis of CVD. Its molecular pathogenesis involves activation of pro-inflammatory and pro-oxidant pathways, along with an imbalance of endothelial vasoactive products. In women, vascular aging is closely interrelated with ovarian endocrine function and hormonal influences. This thesis reinforces evidence of augmented ET-1 pathway together with deficient NO modulation of resistance artery function in a model of aging in female rats. I am demonstrating novel evidence that the upstream dysfunction of ET-1 and NO generating enzymes, MMP and nNOS, is imparted in the altered vasoactive mechanisms associated with aging and/or ovarian deficiency. Vasoprotective potential of estrogen was evaluated in young and aging rats that underwent ovariectomy followed by the long-term E2 replacement. My experimental findings indicate that E2 signaling is impaired in aging, in part due to dysfunction at the level of its mediators, MMP and nNOS. The primary findings of the thesis, together with hypothetical missing links discussed in this chapter, are summarized in Figure 6-1.

6.2 ESTROGEN-DEPENDENT VASOACTIVE PATHWAYS IN A MODEL OF FEMALE AGING

To understand the pathogenesis underlying vascular dysfunction in postmenopausal women we used a rat model of ovarian deficiency. In this model, we examined the mechanisms whereby estrogen (endogenous and exogenous) regulates vascular function, both acutely and in the long-term. To address the aging aspect, interventions were performed in young female rats and the older counterparts who had reached reproductive senescence (i.e. menopause phenotype). Previous studies conducted in our laboratory using a similar model of menopause described a number of vascular alterations that mimic those found in postmenopausal women. In particular, aging estrogen deficient rats develop a pro-inflammatory phenotype with increased circulating TNF levels, activation of renin-angiotensin system and reduced NO bioavailability.[1-3] In addition, increased release of MMP-2 from endothelial cells stimulated with inflammatory factors was demonstrated, although the implications for vessel function were unknown.[4] Altogether, the above work presented in a PhD thesis by I Arenas set some of the foundations for my studies.

6.2.1 MMP and Vascular Constriction

In my work, dysfunction of small arteries in aging ovarian deficient rats was evident as an increased vasoconstriction through the ET-1 pathway, which was due to increased bET-1 processing by MMP. Indeed, the results of experiments

shown in chapters 2 and 3 demonstrated that MMP inhibition (with GM6001) attenuated vascular response to bET-1 (the protease substrate), while it did not have an effect on the ET-1 (the end product) (Appendix A-2). This MMP-mediated vasoconstriction was not observed in young cycling females, whereas in aging it could result from a greater MMP-2 activity detected in vessel tissues. In rats treated with E2 or TNF inhibitor (Etanercept), vascular MMP-2 activity was reduced, corresponding with the previous findings by Arenas et al.,[2, 4] and this was associated with normalized bET-1 contractility. Despite these seemingly favourable outcomes, there was a further increase of MMP-mediated component in big ET-1 response in aging E2 treated rats (compared to placebo or TNF antagonism). The increased acute MMP action resulting from E2 replacement in aging animals (which wasn't present in the young) is a previously unknown phenomenon. Although at this time its significance is difficult to ascertain, it supports the "timing hypothesis" and emphasizes the need for better understanding of impact of aging on E2 signaling.

Further studies may elucidate mechanisms whereby E2 regulates MMPs. Although in our model TNF did not appear to link E2 and MMP activity, the role of other inflammatory factors cannot be discounted since estrogen is a global regulator of immune function. In addition, direct genomic modulation of MMP promoter may not be ruled out. Although it hasn't been shown to contain the complete ERE sequence, the alternative mechanisms of gene regulation by E2 are known (Figure 1-2). Moreover, posttranslational MMP activation may explain its rapid functional effects despite the reduced expression in E2 treated aging

vasculature. For example, TIMP- and/or ROS-mediated events may be involved in acute regulation of MMP activity downstream of E2.[5, 6] Further understanding of the specific ER and MMP type involved in this E2 signaling pathway in the aging vascular system may push the translational potential of this work. Interestingly, besides aging, MMP-mediated vasoactive events have recently been implicated in a number of acute CVD, including preeclampsia, hypertension and septic shock.[7-9] Although, most of the above referenced work is at an early experimental stage and the specific MMP-mediated mechanims are not well characterized yet.

6.2.2 nNOS and Vascular Relaxation

Along with the augmented ET-1 system, NO deficiency is the cornerstone in the pathogenesis of CVD. It may result from decreased NOS-dependent production or increased consumption by ROS. In my joint studies (Chapters 4 and 5), we identified nNOS in endothelial cells, where it appeared a viable source of NO, along with eNOS, its classical and long assumed the only constitutive source. Together with S Chakrabarti, we demonstrated that E2 rapidly stimulated nNOS activity in HUVEC; whereas at the whole vessel level, selective nNOS inhibition attenuated vasorelaxation elicited by E2. Aging and/or ovarian deficiency in animals resulted in a loss of nNOS contribution to acute E2-induced vascular relaxation, and this was not restored in E2-replaced animals regardless of age. Although we found no significant change in total nNOS expression in vascular tissue with age or Ovx, there was a dramatic shift in its enzymatic

function towards superoxide production in these conditions. Interestingly, in E2-treated animals nNOS was not a source of vascular superoxide. Taken together, although E2 replacement appeared to improve nNOS coupling and reduce superoxide production in aging, it did not restore nNOS-mediated vascular relaxation.

Since the vasoactive role of nNOS was absent in Ovx state and was not re-established by E2 replacement, other ovarian hormones or factors must be considered in nNOS regulation. A series of recent studies reported the importance of intact ovarian cycle with the physiological hormonal fluctuations for nitrergic system in specific brain regions regulating sexual behavior. The authors found that both estrogen and progesterone are necessary and act synergistically in regulating brain nNOS and NO-mediated mating function in cycling female mice.[10] In addition, various sex hormone receptors (including estrogen, progesterone and androgen receptors) colocalized with nNOS in both female and male rodents.[11] Interestingly, there was a sex dimorphism in nNOS expression, which cyclically fluctuated in females, versus males who had a higher number of nNOS immunoreactive neurons relative to any phase of estrous cycle.[11] In summary, it is likely that nNOS-mediated vascular relaxation observed in our studies also requires intact ovarian function. Although E2 replacement alone was not sufficient to improve this aspect of vessel function, combined hormone replacement regimens or SERM might be considered for future experimentation. The fact that E2 treatment had some favourable effects, such as reducing nNOS-

dependent superoxide levels, may merit exploration of other potential roles of this enzyme in our model, such as a regulator of vascular inflammation.

6.2.3 Cross-Talk Between MMP and nNOS in Vascular Regulation

As detailed in the background to this dissertation (section 1.4.2), ET-1 and NO vascular pathways interact at many levels.[12] Although the potential cross-talk at their enzyme level has not been directly examined in my study model, it is an interesting speculation. In fact, a few recent studies suggested interactions between NOS and MMP in different experimental settings and models. Gu et al. have shown colocalization of nNOS and active MMP-9 during cerebral ischemia in a rodent model of stroke.[13] *In vitro*, NO directly activated recombinant proMMP-9 via S-nitrosylation, whereas exposure of cerebrocortical cell culture to this S-NO-MMP-9 induced neuronal death.[13] Altogether, this study proposed nNOS/NO mediated MMP activation as a signaling pathway of a potential merit in the development of new therapies for stroke or other vascular disorders associated with nitrosative/oxidative stress.[13] Relative to my model, ROS/RNS derived from uncoupled nNOS in aging (or CVD settings) may lead to MMP activation via the mechanisms described in the background chapter (section 1.5.2). In particular, we detected higher levels of nitrotyrosinated proteins in vascular tissue from aging and Ovx animals (data shown in Appendix A-6). Such a cumulative damage as a footprint of short-lived peroxynitrite suggests its greater presence in the aging vasculature, where it may play a role in rapid modulation of MMP activity.

Another recent study has shown colocalization of MMP-2 with eNOS and its cofactor HSP90 in endothelial cells. This work by Nagareddy et al. suggested MMP-mediated HSP90 cleavage and degradation as a mechanism responsible for reduced NO synthesis, endothelial dysfunction and hypertension in fructose fed hypertensive rats.[14]

Taking into account these latest pieces of evidence, ET-1 and NO vasoactive pathways may interact at the upstream level, via MMP-nNOS dialogue. The latter may be important for understanding CVD in women, as both enzymes are regulated by estrogen. Figure 6-1 illustrates these vasoactive pathways in my model of female aging.

6.3 THERAPEUTIC POTENTIAL: PROS AND CONS

Given that alterations in the NO and ET pathways are associated with the pathogenesis of CVD, it is not surprising that their pharmacological targeting has been attempted, and is likely to yield effective CV therapies. Indeed, a few therapeutic approaches are currently available to potentiate NO-dependent vasodilation: such as NO donors (e.g., nitrates) and cGMP-specific phosphodiesterase inhibitors (e.g., sildenafil), or to ameliorate ET-mediated vasoconstriction: such as ETRs antagonists (e.g., bosentan). However, there are a number of limitations associated with the above therapies including considerable side-effects and the absence of long-term mortality reduction benefits. Moreover, as highlighted in the recent review by S Bourque et al., ETRs

antagonism might not be effective to reverse late stage vascular remodelling caused by prolonged exposure to ET-1, instead it might better be suited for CVD prophylaxis or early endothelial dysfunction.[12] Alternatively, molecular interventions could potentially be explored at the upstream level, i.e. biosynthesis of these vasoactive mediators. Moreover, with better understanding of vasoprotective pathways regulated by estrogen, more specific ideas/targets for biopharm engineering and modeling could emerge. Taking into consideration the results of this thesis, two vascular enzymes, MMP and nNOS, may merit such attention. In my study designs, the role of these enzymes was evaluated using an *ex vivo* inhibition approach with synthetic inhibitors applied to isolated vessels. In addition, *in vivo* modulation of MMP or nNOS was attempted in whole animals via administration of E2, their natural regulator. The current status of pre-clinical and early clinical work with MMP and nNOS inhibitors is discussed below.

6.3.1 Targeting MMP

As soon as abnormal MMP activity was linked to CVD pathogenesis, a great promise was placed at the potential MMP-based interventions. In the past years, many preclinical and clinical studies were focused on ECM manipulation in the field of arthritis, malignancy and periodontal disease. Some led to the US FDA approved new drugs such as Periostat, an MMP inhibiting sub-antimicrobal dose formulation of doxycycline.[15] Since MMPs are now viewed as signaling proteases acting on a broader range of substrates and quicker time scale, it may explain some of the challenges with attempts of enzyme inhibition. Some of the

current deficits in our knowledge are whether complete MMP inhibition is desirable or whether there is an optimal window for intervention so not to interfere with the adaptive aspects of tissue remodelling. Also, understanding functional specialization of individual MMP members could set a basis for developing more targeted pharmacological tools in CVD.[16-18] Although a variety of chemical compounds and molecular techniques have been developed to achieve MMP inhibition in laboratory settings, few (e.g., doxycycline) are potentially suitable for *in vivo* use. There is evidence that doxycycline prevents maladaptive vascular remodelling in a rat model of 2K-1C hypertension, particularly via MMP-2 inhibition.[19, 20] Another study demonstrated differences in the effect of doxycycline in conduit versus small arteries in rats with L-NAME-induced hypertension. Whereas this drug inhibited MMP-2 in both types of arteries, it prevented hypertrophic remodelling in aorta but not eutrophic remodelling in mesenteric arteries.[21] Interestingly, some of the conventional antihypertensive medications (e.g., from the classes of angiotensin-converting enzyme inhibitors, calcium channel blockers, and diuretics) in addition to their blood pressure lowering effects, are also found to downregulate MMPs that may provide an additional "off-label" CV protection.[22-24] Moreover, recent pilot clinical trials suggested that MMP inhibition may exert systemic anti-inflammatory effects and prevent CV events.[25, 26]

6.3.2 Targeting nNOS

Increased expression and/or activity of nNOS and excess NO in the central nervous system have been associated with many disorders of neurological nature such as migraines, seizures and ischemic neuronal injury.[27] Experimentally, in animal models of stroke, selective nNOS inhibition or nNOS gene knockout reduce the infarct size.[28-30] Rapid increase in information on the physiological and pathological role of brain nNOS during only the two decades from its discovery resulted in the promising therapeutic agents. Indeed, a number of nNOS inhibitors (first developed by NeurAxon, www.neuraxon.com) have been tested as far as phase II and phase I clinical trials for treatment of central nervous system conditions. If better understood, vascular nNOS may prove its significant role in health and/or disease, which may open novel therapeutic opportunities for CVD.

6.4 GENERAL LIMITATIONS AND FUTURE DIRECTIONS

The importance of the specific vasoactive pathways in female vascular regulation has been concluded from my studies conducted in isolated vessel preparations. Whereas this work provides valuable mechanistic insights, the *in vivo* regulation of vascular function is more complex and integrates neural inputs together with a host of interacting endocrine, paracrine and autocrine factors. Following a better understanding of cellular mechanisms by which estrogen regulates its downstream mediators, the studies can be extended to whole

animal interventions targeting the MMP- and nNOS-mediated mechanisms. To pursue this, there are a number of experimental tools developed and currently commercially accessible, including synthetic MMP and nNOS inhibitors suitable for *in vivo* administration, and knock-out animals.

I used small mesenteric arteries as a representative of resistance vasculature for assessment of vascular function. Whereas resistance artery tone is the key determinant of systemic blood pressure, NO is only a component of vascular regulation in this type of arteries, where other endothelial factors (such as EDHF) may also play important roles. Therefore, it might be appropriate to evaluate functional role of nNOS in a highly NO-dependent vessel such as aorta, where nNOS protein expression and nNOS-dependent superoxide generation were measured.

E2 used in my studies is a non-selective ER agonist. Determining the specific receptor type, which mediated rapid and longer-term vasoactive actions of E2 described in chapters 2 to 5 might be the important further steps. Interestingly, a widely used, experimentally and clinically, ERα/ERβ antagonist ICI 182780 (a.k.a. fulvestrant) was recently found to be a simultaneous GPER agonist,[31] i.e. in fact a SERM. Therefore, many of the previous conclusions based on the use of ICI 182780 need to be re-interpreted carefully in the light of this new knowledge. For example, it has been reported that acute vasodilatory action of E2 in male rat mesenteric arteries was ER-independent, since it was not reduced in the presence of ICI 182780.[32] Although there is a probability that E2 acts through yet-unknown receptor, alternatively the GPER-mediated vascular

effects must not be discounted and is a promising new venue for research. An intriguing preliminary observation made in our HUVEC model suggests that GPER may mediate rapid activation of nNOS in endothelial cells. Indeed, pretreatment of the cells with ICI 182780 in the absence of E2 resulted in increased activating site phosphorylation of nNOS (Figure 4-1C). Thus, targeting the correct ER may allow the achievement of desired effects while minimizing the risks of non-selective estrogenic actions.

Beside the specific focus of my thesis on MMP- and nNOS-mediated vasoactive mechanisms, other estrogen-dependent vascular pathways may be impaired during aging. Indeed, whereas direct vascular responses elicited by estrogen have been well characterized (as reviewed in the introduction), little is known how these are affected by oxidative stress and inflammation - the practical issues encountered in clinical scenarios.

Although my research interests predominantly included aging models in the female, the questions often arise whether the observations are truly unique to the female sex. Indeed, neither MMP nor nNOS contributed to the vasoactive pathways in intact male rats, irrespective of their age (Appendix A-1 and Figure 4-5). Nonetheless, since the interest in designing estrogen-based therapeutic applications for both sexes is high, curiosity-driven experiments with estrogen supplementation might be considered in age-matched male species. Interesting results could be anticipated given that male animlas equally possess all the signaling machinery, including similar expression of ERs in vascular tissue (data described in Chapter 4, shown in Appendix A-3). For example, based on some

encouraging animal data, recent small human trials in male patients with coronary artery disease evaluated the clinical efficacy of local E2 delivery in a form of a coating agent for endovascular stents.[33]

6.5 SIGNIFICANCE OF THE THESIS RESULTS

Considering the forecasting of global aging of the population, there is a critical need in understanding mechanisms of aging together with its CV implications. In particular, understanding the impact of menopause on women's CV health is not yet sufficient to meet the current and future health care needs. Our results support the complex role of estrogen in vascular regulation that is age-dependent. In fact, it is not presently clear whether menopause is meant as a protective mechanism to prevent estrogen-induced damage in aging, or, in contrast, it should be viewed as a lack of timely adaptations to a rapidly increasing life span in humans. The results presented in this thesis suggest new roles for old enzymes that appear to mediate vascular actions of estrogen differentially in young versus aging conditions. This work has implications for understanding the complexities behind the hormone replacement therapies, and hopefully, will aid in the future vascular research.

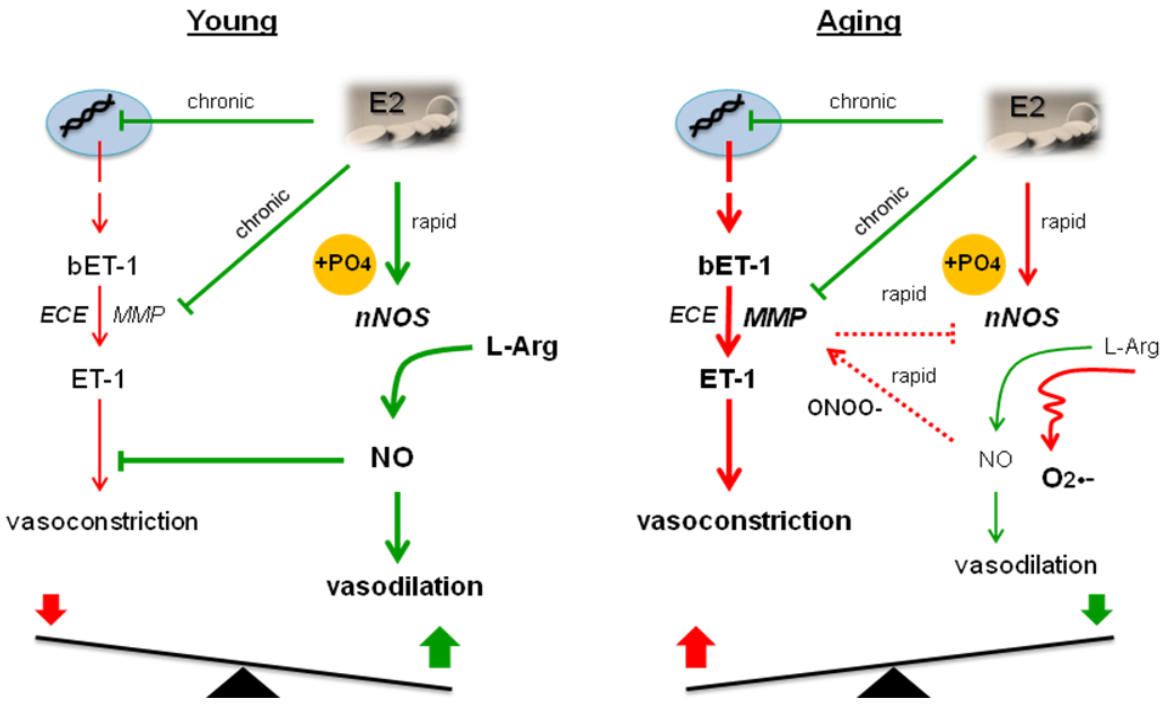

Figure 6-1. MMP and nNOS mediated vasoactive pathways in female aging: implications for hormone replacement

In the young vascular system, the balance between ET-1 and NO favors anti-hypertensive phenotype. Estrogens act to suppress ET-1 at various levels, including conversion of bET-1. In addition, estrogens stimulate NO production (which itself is an inhibitor of ET-1 signaling) via a novel mechanism - rapid activation of nNOS.

In aging, there is a general hyperactivation of ET-1 system, including MMP-mediated conversion of bET-1. Exogenous E2 can suppress long-term MMP expression, however further potentiates acute MMP-dependent bET-1 cleavage. One potential cause of rapid MMP activation is ROS/RNS derived from E2-stimulated uncoupled nNOS. MMP activity may itself contribute to NOS dysfunction, via for example proteolytic degradation of its chaperone HSP90 (as has been shown for eNOS). Altogether, the balance in vascular tone is shifted towards more vasoconstriction. In this scenario, E2 signaling is not selective enough to restore the altered vascular function.

REFERENCES

1. Arenas IA, Armstrong SJ, Xu Y, Davidge ST. Chronic tumor necrosis factor-alpha inhibition enhances NO modulation of vascular function in estrogen-deficient rats. *Hypertension.* 2005;46(1):76-81.

2. Arenas IA, Armstrong SJ, Xu Y, Davidge ST. Tumor necrosis factor-alpha and vascular angiotensin II in estrogen-deficient rats. *Hypertension.* 2006;48(3):497-503.

3. Arenas IA, Xu Y, Davidge ST. Age-associated impairment in vasorelaxation to fluid shear stress in the female vasculature is improved by TNF-alpha antagonism. *American journal of physiology.* 2006;290(3):H1259-1263.

4. Arenas IA, Xu Y, Lopez-Jaramillo P, Davidge ST. Angiotensin II-induced MMP-2 release from endothelial cells is mediated by TNF-alpha. *Am J Physiol Cell Physiol.* 2004;286(4):C779-784.

5. Voloshenyuk TG, Gardner JD. Estrogen improves TIMP-MMP balance and collagen distribution in volume-overloaded hearts of ovariectomized females. *Am J Physiol Regul Integr Comp Physiol.*299(2):R683-693.

6. Lam KK, Cheng PY, Hsiao G, Chen SY, Shen HH, Yen MH, Lee YM. Estrogen deficiency-induced alterations of vascular MMP-2, MT1-MMP, and TIMP-2 in ovariectomized rats. *American journal of hypertension.* 2009;22(1):27-34.

7. Estrada-Gutierrez G, Cappello RE, Mishra N, Romero R, Strauss JF, 3rd, Walsh SW. Increased expression of matrix metalloproteinase-1 in systemic vessels of preeclamptic women: a critical mediator of vascular dysfunction. *The American journal of pathology.*178(1):451-460.

8. Cena J, Lalu MM, Rosenfelt C, Schulz R. Endothelial dependence of matrix metalloproteinase-mediated vascular hyporeactivity caused by lipopolysaccharide. *European journal of pharmacology.* 2008;582(1-3):116-122.

9. Odenbach J, Wang X, Cooper S, Chow FL, Oka T, Lopaschuk G, Kassiri Z, Fernandez-Patron C. MMP-2 mediates angiotensin II-induced

hypertension under the transcriptional control of MMP-7 and TACE. *Hypertension.*57(1):123-130.

10. Martini M, Pradotto M, Panzica G. Synergic effects of estradiol and progesterone on regulation of the hypothalamic neuronal nitric oxide synthase expression in ovariectomized mice. *Brain research.* 2011;1404:1-9.

11. Sica M, Martini M, Viglietti-Panzica C, Panzica G. Estrous cycle influences the expression of neuronal nitric oxide synthase in the hypothalamus and limbic system of female mice. *BMC neuroscience.* 2009;10:78.

12. Bourque SL, Davidge ST, Adams MA. The interaction between endothelin-1 and nitric oxide in the vasculature: new perspectives. *Am J Physiol Regul Integr Comp Physiol.* 2011;300(6):R1288-1295.

13. Gu Z, Kaul M, Yan B, Kridel SJ, Cui J, Strongin A, Smith JW, Liddington RC, Lipton SA. S-nitrosylation of matrix metalloproteinases: signaling pathway to neuronal cell death. *Science (New York, N.Y.* 2002;297(5584):1186-1190.

14. Nagareddy P, Rajput P, Vasudevan H, McClure B, Kumar U, Macleod K, McNeill J. Inhibition of matrix metalloproteinase-2 improves endothelial function and prevents hypertension in insulin resistant rats. *British journal of pharmacology.* 2011.

15. Caton J, Ryan ME. Clinical studies on the management of periodontal diseases utilizing subantimicrobial dose doxycycline (SDD). *Pharmacol Res.* 2011;63(2):114-120.

16. Johnson JL, Devel L, Czarny B, George SJ, Jackson CL, Rogakos V, Beau F, Yiotakis A, Newby AC, Dive V. A selective matrix metalloproteinase-12 inhibitor retards atherosclerotic plaque development in apolipoprotein E-knockout mice. *Arteriosclerosis, thrombosis, and vascular biology.* 2011;31(3):528-535.

17. Wang X, Chow FL, Oka T, Hao L, Lopez-Campistrous A, Kelly S, Cooper S, Odenbach J, Finegan BA, Schulz R, Kassiri Z, Lopaschuk GD, Fernandez-Patron C. Matrix metalloproteinase-7 and ADAM-12 (a disintegrin and metalloproteinase-12) define a signaling axis in agonist-induced hypertension and cardiac hypertrophy. *Circulation.* 2009;119(18):2480-2489.

18. Odenbach J, Wang X, Cooper S, Chow FL, Oka T, Lopaschuk G, Kassiri Z, Fernandez-Patron C. MMP-2 mediates angiotensin II-induced hypertension under the transcriptional control of MMP-7 and TACE. *Hypertension.* 2011;57(1):123-130.

19. Castro MM, Rizzi E, Figueiredo-Lopes L, Fernandes K, Bendhack LM, Pitol DL, Gerlach RF, Tanus-Santos JE. Metalloproteinase inhibition ameliorates hypertension and prevents vascular dysfunction and remodeling in renovascular hypertensive rats. *Atherosclerosis.* 2008;198(2):320-331.

20. Castro MM, Rizzi E, Prado CM, Rossi MA, Tanus-Santos JE, Gerlach RF. Imbalance between matrix metalloproteinases and tissue inhibitor of metalloproteinases in hypertensive vascular remodeling. *Matrix Biol.* 2010;29(3):194-201.

21. Bouvet C, Gilbert LA, Girardot D, deBlois D, Moreau P. Different involvement of extracellular matrix components in small and large arteries during chronic NO synthase inhibition. *Hypertension.* 2005;45(3):432-437.

22. Martinez ML, Rizzi E, Castro MM, Fernandes K, Bendhack LM, Gerlach RF, Tanus-Santos JE. Lercanidipine decreases vascular matrix metalloproteinase-2 activity and protects against vascular dysfunction in diabetic rats. *European journal of pharmacology.* 2008;599(1-3):110-116.

23. Yamamoto D, Takai S, Miyazaki M. Inhibitory profiles of captopril on matrix metalloproteinase-9 activity. *European journal of pharmacology.* 2008;588(2-3):277-279.

24. Ceron CS, Castro MM, Rizzi E, Montenegro MF, Fontana V, Salgado MC, Gerlach RF, Tanus-Santos JE. Spironolactone and hydrochlorothiazide exert antioxidant effects and reduce vascular matrix metalloproteinase-2 activity and expression in a model of renovascular hypertension. *British journal of pharmacology.*160(1):77-87.

25. Brown DL, Desai KK, Vakili BA, Nouneh C, Lee HM, Golub LM. Clinical and biochemical results of the metalloproteinase inhibition with subantimicrobial doses of doxycycline to prevent acute coronary syndromes (MIDAS) pilot trial. *Arteriosclerosis, thrombosis, and vascular biology.* 2004;24(4):733-738.

26. Payne JB, Golub LM, Stoner JA, Lee HM, Reinhardt RA, Sorsa T, Slepian MJ. The effect of subantimicrobial-dose-doxycycline periodontal therapy

on serum biomarkers of systemic inflammation: a randomized, double-masked, placebo-controlled clinical trial. *Journal of the American Dental Association (1939).*142(3):262-273.

27. Zhou L, Zhu DY. Neuronal nitric oxide synthase: structure, subcellular localization, regulation, and clinical implications. *Nitric Oxide.* 2009;20(4):223-230.

28. Huang PL. Neuronal and endothelial nitric oxide synthase gene knockout mice. *Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas / Sociedade Brasileira de Biofisica ... [et al.* 1999;32(11):1353-1359.

29. Nanri K, Montecot C, Springhetti V, Seylaz J, Pinard E. The selective inhibitor of neuronal nitric oxide synthase, 7-nitroindazole, reduces the delayed neuronal damage due to forebrain ischemia in rats. *Stroke; a journal of cerebral circulation.* 1998;29(6):1248-1253; discussion 1253-1244.

30. Sun Y, Jin K, Childs JT, Xie L, Mao XO, Greenberg DA. Neuronal nitric oxide synthase and ischemia-induced neurogenesis. *J Cereb Blood Flow Metab.* 2005;25(4):485-492.

31. Meyer MR, Baretella O, Prossnitz ER, Barton M. Dilation of epicardial coronary arteries by the G protein-coupled estrogen receptor agonists G-1 and ICI 182,780. *Pharmacology.*86(1):58-64.

32. Shaw L, Taggart MJ, Austin C. Mechanisms of 17 beta-oestradiol induced vasodilatation in isolated pressurized rat small arteries. *British journal of pharmacology.* 2000;129(3):555-565.

33. Ryu SK, Mahmud E, Tsimikas S. Estrogen-eluting stents. *Journal of cardiovascular translational research.* 2009;2(3):240-244.

APPENDIX A:

SUPPLEMENTARY DATA

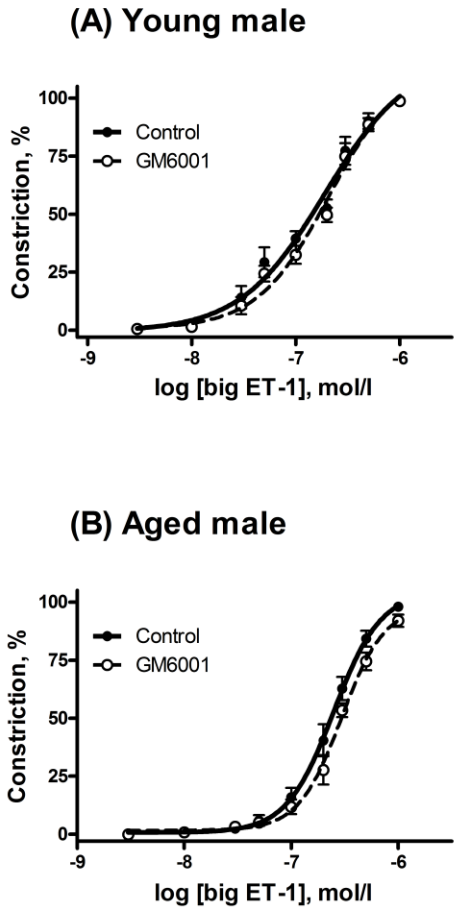

Figure A-1. In males, MMP does not modulate vasoconstriction to big ET-1[*†]

Dose-response of small mesenteric arteries to exogenous big ET-1 was assessed in the absence (solid circles) or presence (open circles) of MMP inhibitor, GM6001 (10 µmol/l) using a pressure myograph. Vessels were isolated from young (4 months old) or aged (14 months old) male Sprague Dawley rats. Data presented as means ± SEM, n=5-10/group.

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[*] *Experiments shown in Panel A were conducted with big ET-1 purchased from Sigma, and in Panel B – with big ET-1 from American Peptide, which could have contributed to differences in vascular sensitivity to the peptide between young and aged animals.*

[†] *This figure complements data presented in Chapter 2 and Chapter 3.*

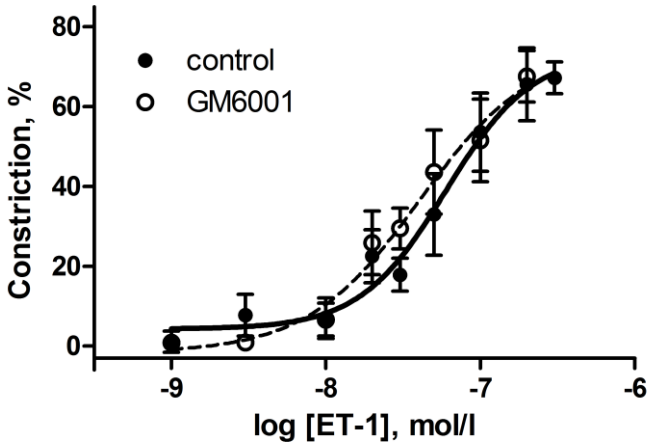

Figure A-2. MMP does not alter vascular response to ET-1 (unlike big ET-1, Figure 2-2) in aging female rats[*†]

Dose-dependent constriction of mesenteric arteries isolated from aging (14 months old) female Sprague Dawley rats to exogenously added ET-$1_{[1-21]}$. Vascular responses were examined in the absence (solid circles) or presence (open circles) of MMP inhibitor, GM6001 (10 µmol/l) on pressure myograph. Data presented as means ± SEM, n=4 (*p*=ns).

[*] *This figure complements data presented in Chapter 2.*

[†] *These pressure myograph experiments were conducted by Jude Morton.*

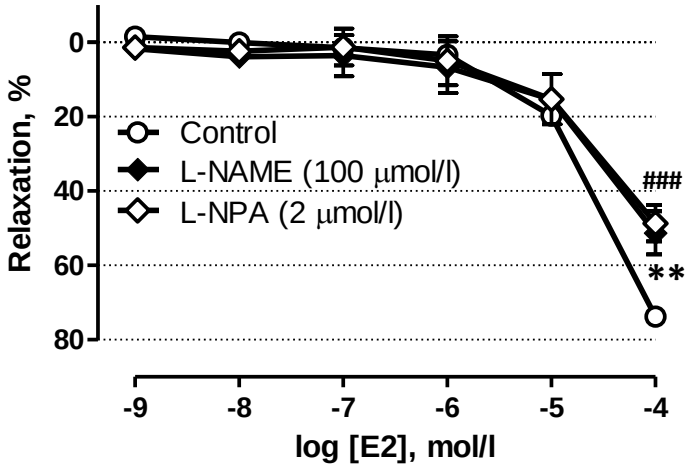

Figure A-3. Effect of nNOS inhibition on E2 relaxation in endothelium-denuded arteries from young control female rats[*]

Dose-dependent relaxation to E2 was not affected in the presence of L-NAME or L-NPA until the higher concentration of E2 (100 μmol/l) was reached. Data presented as means ± SEM, n=4. ** $p<0.01$ for L-NAME treated vessel vs control. ### $p<0.001$ for L-NPA treated vessel vs control.

[*] *Thisfigure complements data presented in Chapter 4.*

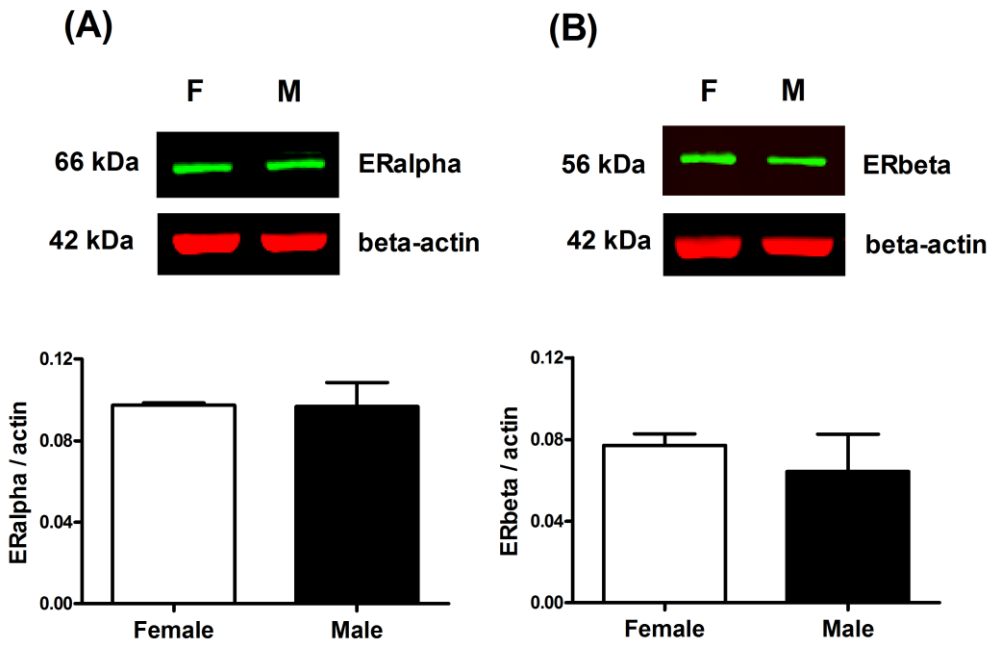

Figure A-4. Vascular expression of estrogen receptors does not differ between sexes[*†]

Protein was extracted from thoracic aortas obtained from female (F) and male (M) Sprague Dawley rats (3-4 months old). Representative immunoblots are shown. The expression of ERα (Panel A) and ERβ (Panel B) were normalized to the corresponding actin bands. Data presented as means ± SEM, n=3 (*p*=ns).

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[*] *This figure complements data presented in Chapter 4.*

[†] *The western blotting was performed by Yanyan Jiang.*

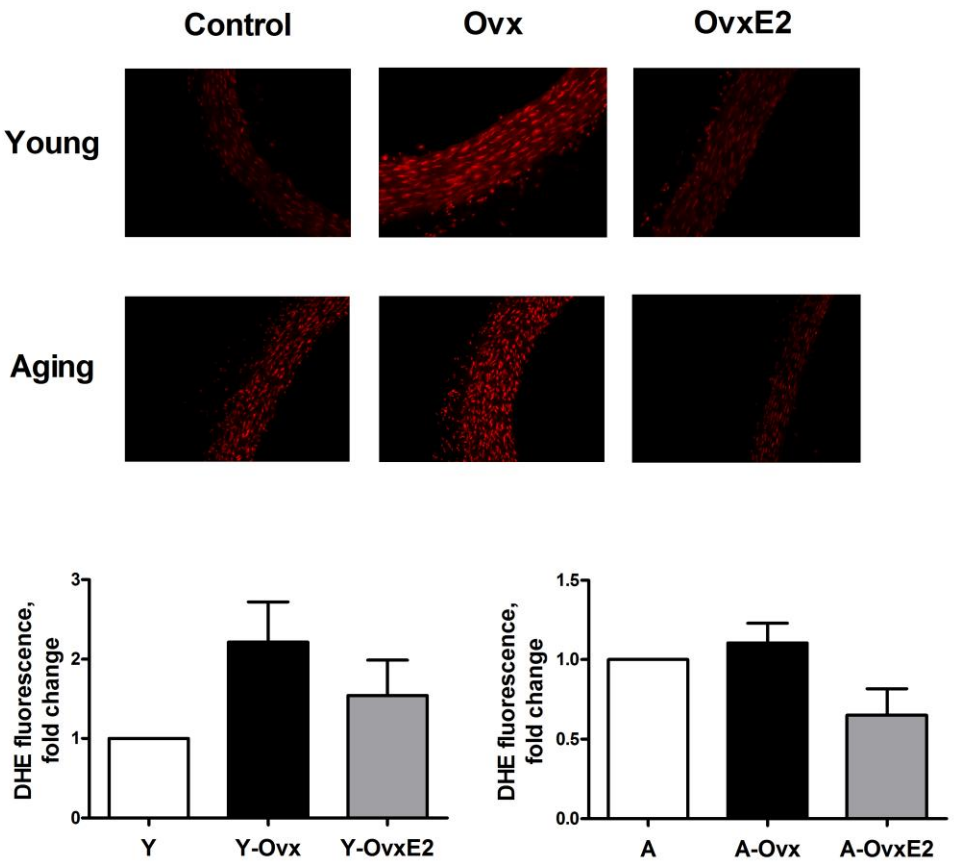

Figure A-5. Vascular superoxide production in aging and Ovx[*]

Superoxide levels were measured by DHE fluorescence (MFI) in the cross-sections of rat thoracic aortas. Impact of Ovx and E2 treatment was assessed in young (Y) and aging (A) animals. Representative images are shown. Data were expressed as fold change over the control animals of respective age and presented as means ± SEM, n=5 rats/group. Young groups: *p*=ns, aging groups: *p*<0.05 (one-way ANOVA).

[*] *This figure complements data presented in Chapter 5 (Figure 5-5).*

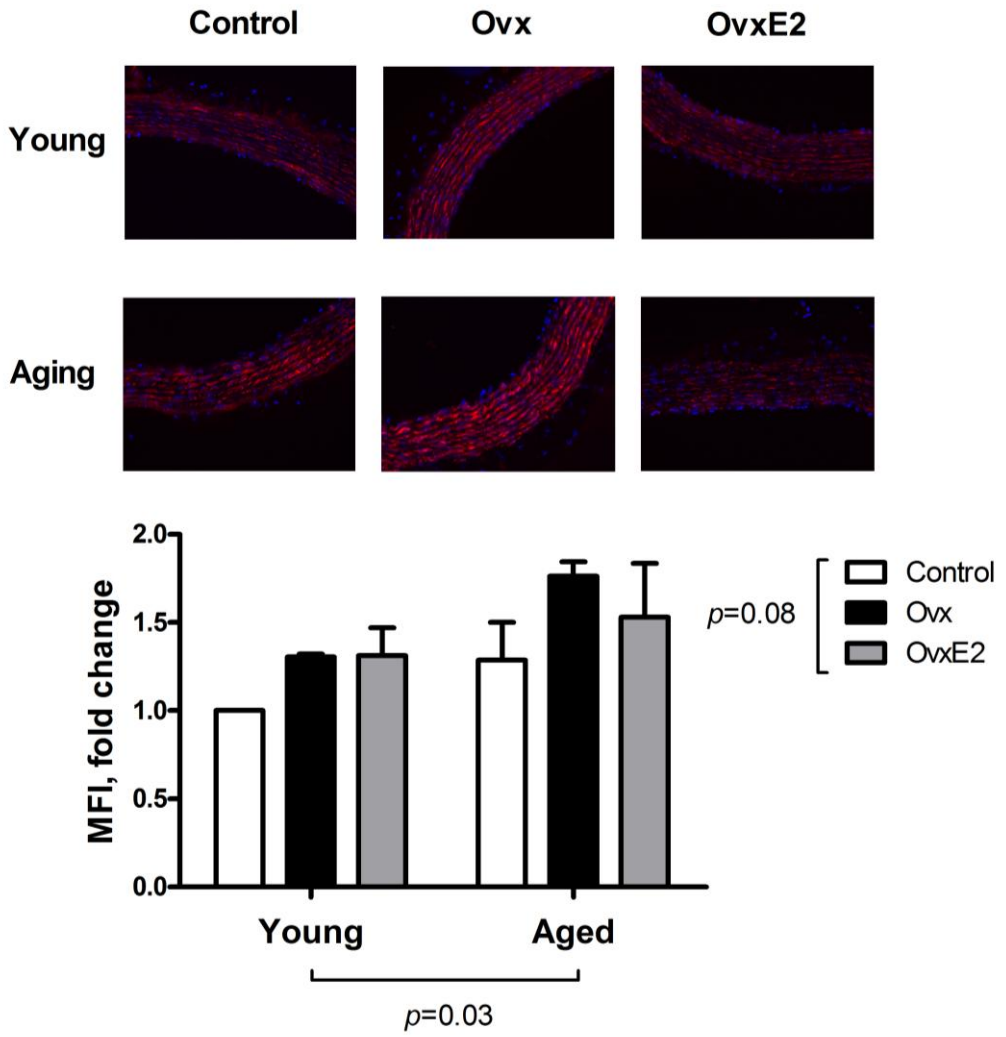

Figure A-6. Vascular nitrotyrosine levels in aging and Ovx

Nitrotyrosinated proteins were detected in the cross-sections of rat thoracic aortas immunostained with the primary anti-nitrotyrosine mouse monoclonal antibodies (Abcam; 1:200), followed by the secondary goat anti-mouse IgG Alexa Fluor 546 (red) (Invitrogen; 1:200). Vascular cells nuclei were visualized with DAPI stain. Representative images are shown. MFI were expressed as fold change over the young control. The results of 4-6 independent experiments were summarized as means ± SEM. *p* values are shown (two-way ANOVA).

APPENDIX B:

ABSTRACT FROM RELATED COAUTHORED MANUSCRIPT

17beta-Estradiol Induces Protein S-nitrosylation in the Endothelium

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Manuscript has been published in Cardiovascular Research 2010;85(4):796-805

Estrogen induces nitric oxide (NO) in the endothelium and appears to protect against inflammation and atherosclerosis. NO can induce post-translational protein modifications such as cysteine S-nitrosylation in the cellular proteins, which may exert anti-inflammatory effects. However, whether estrogen can induce protein S-nitrosylation in the endothelium is not known. Given this background, we investigated the role of 17beta-estradiol (E2), the major form of estrogen in the body, on endothelial protein S-nitrosylation.

Experiments were performed in human umbilical vein endothelial cells (HUVEC). S-nitrosylation was detected by immunostaining for nitrosocysteine and further confirmed by biotin switch method. Ovariectomized 12-month-old Sprague Dawley rats with/without E2 supplementation were used for *in vivo* validation of findings. We found that physiologically relevant doses of E2 increased protein S-nitrosylation in HUVECs through estrogen receptor-alpha (ERα) and endothelial nitric oxide synthase (eNOS). Interestingly, specific agonists for both ERα and ERβ increased eNOS protein expression, while only the former could activate eNOS through phosphorylation. S-nitrosylation by E2 prevented angiotensin II-induced upregulation of intercellular cell adhesion molecule-1, suggesting a potential anti-inflammatory mechanism. Finally, we showed that exogenous E2 could increase endothelial S-nitrosylation *in vivo* in a rat model.

Our results demonstrate for the first time that E2 increases protein S-nitrosylation in the vascular endothelium, which might be a novel pathway to mediate the protective effects on the vasculature.