PHYSIOLOGICAL FACTORS THAT MODULATE VASCULAR FUNCTION: STATES OF ENDOTHELIAL DYSFUNCTION AND THERAPEUTIC INTERVENTIONS

A Dissertation

by

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ABSTRACT

This dissertation investigated the role of two therapeutic interventions (exercise training and hormone replacement therapy) on two different states of endothelial dysfunction, chronic coronary occlusion and aging. Despite remarkable evidence for the therapeutic benefits of physical activity, the mechanisms by which regular exercise improves vascular function in the setting of coronary artery disease are not fully understood. Similarly, the effects of aging and hormone replacement therapy on vascular function are often paradoxical and poorly understood. Thus, the first project utilized a model of chronic coronary artery occlusion to evaluate the effects of exercise training on cellular and molecular adaptations of collateral-dependent coronary vasculature compared to the nonoccluded control. This study provided new evidence that exercise training concomitantly enhanced the contributions of multiple vasodilator mechanisms, including nitric oxide, prostacyclin and BK_{Ca} channels to vascular function in the ischemic heart. Increased contribution of multiple vasodilator signaling pathways after exercise training appears to promote compensation or redundancy to ensure adequate vasodilation and coronary vascular blood flow. The second project utilized a model of aging to evaluate the interactive effects of age and hormone replacement therapy on the cellular and molecular mechanisms underlying the regulation of cerebrovascular function. Although the mechanisms underlying the beneficial effects of estrogen on cerebrovascular function have been studied at length, the mechanisms responsible for age-dependent deleterious effects of estrogen are largely unknown. The results of this study revealed

that estrogen exerts divergent effects on the cerebrovasculature with advancing age. In younger females, estrogen replacement treatment is beneficial, attenuating vasoconstriction primarily by the COX-1 dependent prostanoid pathway and increased PGI_2 production. In contrast, in older reproductively senescent females, estrogen augmented vasoconstriction via the COX-2 dependent prostanoid pathway and increased TXA_2 production. A better understanding the mechanisms by which estrogen exerts beneficial versus detrimental effects on the cerebrovasculature may lead to new gender-specific therapeutic agents designed specifically to target the cerebrovascular system and other estrogen-responsive tissues.

DEDICATION

To three amazing science teachers who were integral in fostering my love for science from a very young age- *Mrs. McKenna* (7th and 8th grade science), *Ms. Vitiello and Miss Ledogar* (AP Biology). Their knowledge and passion sparked my scientific curiosity; they inspired, motivated, and helped me to see the world differently.

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NOMENCLATURE

AA	arachidonic acid
ANG II	angiotensin II
ARC	arcuate nucleus
AVPV	anteroventral periventricular nucleus
BK_{Ca}	large-conductance calcium-dependent potassium channel
BST	bed nucleus of the stria terminalis
Ca^{2+}	calcium
CaM	calmodulin
cAMP	cyclic adenosine monophosphate
CBF	cerebral blood flow
CEE	conjugated equine estrogen
cGMP	cyclic guanosine monophosphate
CNP	C-type natriuretic peptide
COX	cyclooxygenase
CYP	cytochrome P450
DAF-FM DA	4-amino-5-methylamino-2’,7’-difluororescein diacetate
DAG	diacylglycerol
DHET	dihydroxyeicosatrienoic acid
ECE	endothelin converting enzyme
EDHF	endothelium derived hyperpolarizing factor

EET	epoxyeicosatrienoic acid
eNOS	endothelial nitric oxide synthase
EP	PGE_2 receptor
ER	estrogen receptor
ERE	estrogen response element
ERK	extracellular signal-related kinase
ET	endothelin
FSH	follicle stimulating hormone
GPR30	G-protein coupled estrogen receptor 30
H_2O_2	hydrogen peroxide
HERS	heart and estrogen/progestin replacement study
HETE	hydroxyeicosatetraenoic acid
HPETE	hydroperoxyeicosatetraenoic acid
HRT	hormone replacement therapy
IBTX	iberiotoxin
INDO	indomethacin
iNOS	inducible nitric oxide synthase
IP	prostacyclin receptor
K^+	potassium
K_{Ca}	calcium-dependent potassium channel
LAD	left anterior descending artery
LCX	left circumflex artery

L-NAME	N^{ω}-nitro-*L*-arginine methyl ester
LO	lipoxygenase
LT	leukotrienes
MA	mature multigravid adult
MCA	middle cerebral artery
MPA	medroxyprogesterone acetate
MPN	medial preoptic nucleus
NADPH	nicotinamide adenine dinucleotide phosphate
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
PCI	percutaneous coronary intervention
p-eNOS	phosphorylated eNOS
PG	prostaglandin
PGD_2	prostaglandin D_2
PGE_2	prostaglandin E_2
6-keto $PGF_{1\alpha}$	6- keto prostaglandin $F_{1\alpha}$, stable metabolite of PGI_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGH_2	prostaglandin H_2
PGI_2	prostacyclin, prostaglandin I_2
PGIS	prostacyclin synthase
PI3K	phosphoinositide 3-kinase

PLA_2	phospholipase A_2
ROS	reactive oxygen species
RS	reproductively senescent
sEH	soluble epoxide hydrolase
TP	thromboxane receptor
TXA_2	thromboxane A_2
TXB_2	thromboxane B_2, stable metabolite of TXA_2
TXS	thromboxane synthase
VEGF	vascular endothelial growth factor
VMN	ventromedial nucleus
VP	arginine vasopressin
WEST	women's estrogen for stroke trial
WHI	women's health initiative

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1. INTRODUCTION

1.1. Vascular endothelium

1.1.1. Endothelial function in health

The vascular endothelium is a single layer of endothelial cells which lines the inner surface of all blood vessels and separates the circulating blood from the surrounding vascular wall. The endothelium not only provides a structural barrier between the circulating blood and surrounding tissues, but is also involved in the regulation of a number of important vascular functions including thrombosis, smooth muscle cell proliferation, vascular tone, cell migration, leukocyte adhesion, and inflammatory responses. Endothelial cells secrete a number of vasoactive mediators that influence vascular structure and function. These mediators regulate local blood flow and systemic blood pressure by acting in a paracrine manner to alter the contractile state of the underlying smooth muscle and the resultant vascular tone (Feletou *et al.*, 2006; Landmesser *et al.*, 2004; Vane *et al.*, 1990; Vanhoutte *et al.*, 2009).

1.1.2. Endothelial-derived vasoactive mediators

In response to mechanical or humoral stimuli, the endothelium releases agents that regulate vascular function (Figure 1). The most common vasodilator substances produced by the endothelium are nitric oxide, prostacyclin (PGI_2), endothelium-derived hyperpolarizing factors (EDHF), and C-type natriuretic peptide (CNP). Common vasoconstrictors include endothelin (ET), angiotensin II (ANG II), thromboxane A_2

(TXA_2), and reactive oxygen species (ROS). The release of nitric oxide and other vascular mediators by endothelial cells can be modulated by both acute and chronic factors. Enhanced secretion or up-regulation of vascular mediators occurs via increased shear stress, hormones, exercise, and diet, while decreased secretion or down-regulation occurs via oxidative stress, smoking, obesity, and vascular diseases (Vanhoutte *et al.*, 2009).

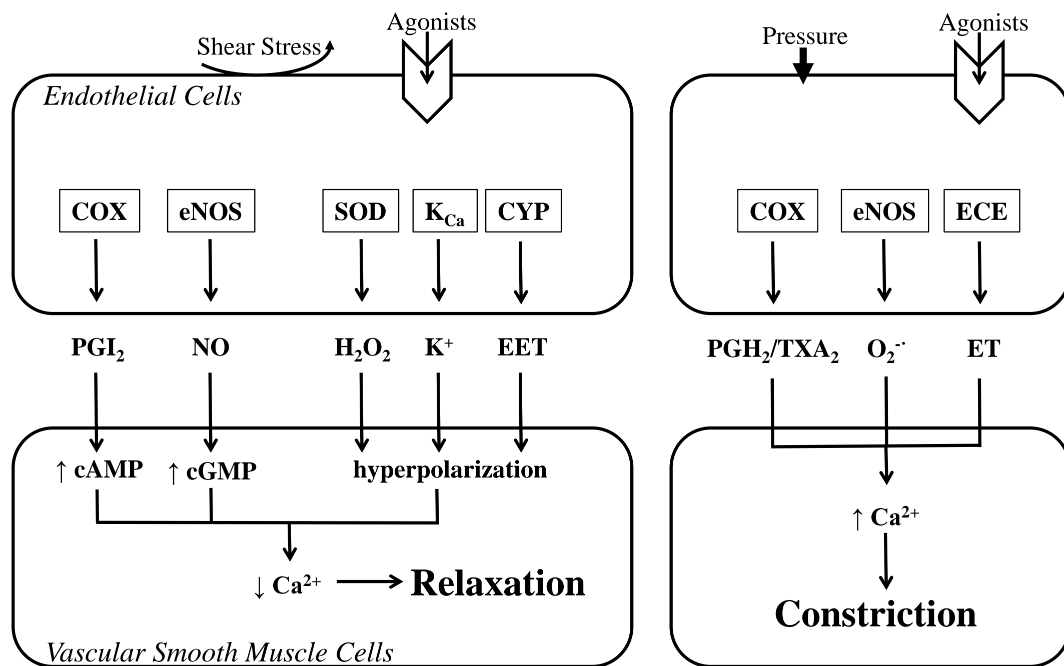

Figure 1. Endothelium-derived relaxing and contracting factors and their effects on vascular smooth muscle.

1.1.2.1. Nitric oxide

One of the most studied endothelium-derived vasodilators is nitric oxide. It is synthesized by the vascular endothelium, and acts as a key modulator of endothelial function, regulating vascular tone and local blood flow. It also inhibits platelet aggregation, limits vascular platelet, leukocyte, and monocyte adhesion, and suppresses vascular smooth muscle cell migration and proliferation (Forstermann *et al.*, 2006; Landmesser *et al.*, 2004; Moncada *et al.*, 1993; Urakami-Harasawa *et al.*, 1997). Nitric oxide is synthesized from L-arginine by a family of nitric oxide synthase (NOS) enzymes. The three distinct isoforms of NOS, endothelial (eNOS), neuronal (nNOS) and inducible (iNOS), differ in both structure and function (Marletta, 1993). The main source of endothelial nitric oxide occurs by the way of constitutively expressed eNOS in endothelial cells, but the enzyme is also found in cardiomyocytes, platelets, and cardiac conduction tissue. Nitric oxide stimulates soluble guanylyl cyclase in the underlying smooth muscle cells, thereby increasing the production of cyclic guanosine monophosphate (cGMP), and causing vasodilation (Forstermann *et al.*, 1994; Sase *et al.*, 1997).

NOS is a multi-domain enzyme containing a C-terminal reductase domain that binds nicotinamide adenine dinucleotide phosphate (NADPH) and a heme-containing N-terminal oxygenase domain that binds the cofactor BH_4, molecular O_2, and the substrate L-arginine. In order to be functional, NOS forms dimers composed of two identical subunits. All isoforms of NOS catalyze electron transfer from NADPH on the C-terminal

to the heme on the N-terminal, consequently activating O_2, and synthesizing nitric oxide. Calcium (Ca^{2+})-induced binding of calmodulin (CaM) to the enzymeincreases electron transfer thus enhancing nitric oxide production (Corson *et al.*, 1996; Forstermann *et al.*, 1994; Forstermann *et al.*, 2006). This Ca^{2+}-dependent stimulation of CaM is not the only regulator of eNOS enzyme activity. (Fleming *et al.*, 1998; Forstermann *et al.*, 2006). It is also regulated by post-translational modification, such as phosphorylation and dephosphorylation of eNOS, and interactions with several regulatory proteins, including heat shock protein 90. Numerous kinases phosphorylate eNOS, resulting in increased or decreased nitric oxide production, including: serine/threonine protein kinase Akt/PKB, AMP-activated protein kinase, protein kinase G, protein kinase A, and CaM-dependent protein kinases II (Boo *et al.*, 2003; Dimmeler *et al.*, 1999; Govers *et al.*, 2001).

1.1.2.2. Arachidonic metabolism

Arachidonic acid (AA) is the most common fatty acid present in the phospholipids of the cell membrane. When the cell membrane is stimulated by physical or humoral stimuli, AA is released by phospholipase A_2 (PLA_2) or by diacylglycerol (DAG) lipase into the cytosol. Here, it undergoes further metabolism via one of the following pathways: 1) the cyclooxygenase (COX) pathway, which produces the prostaglandins (PGs); 2) the lipoxygenase (LO) pathway, which gives rise to the leukotrienes and several hydroperoxides; or 3) the cytochrome P450 (CYP) pathway, which forms the epoxyeicosatrienoic acids (EETs) and ω-hydroxyeicosatetraenoic acid (HETEs) (Capdevila *et al.*, 2000; Needleman *et al.*, 1986) (Figure 2).

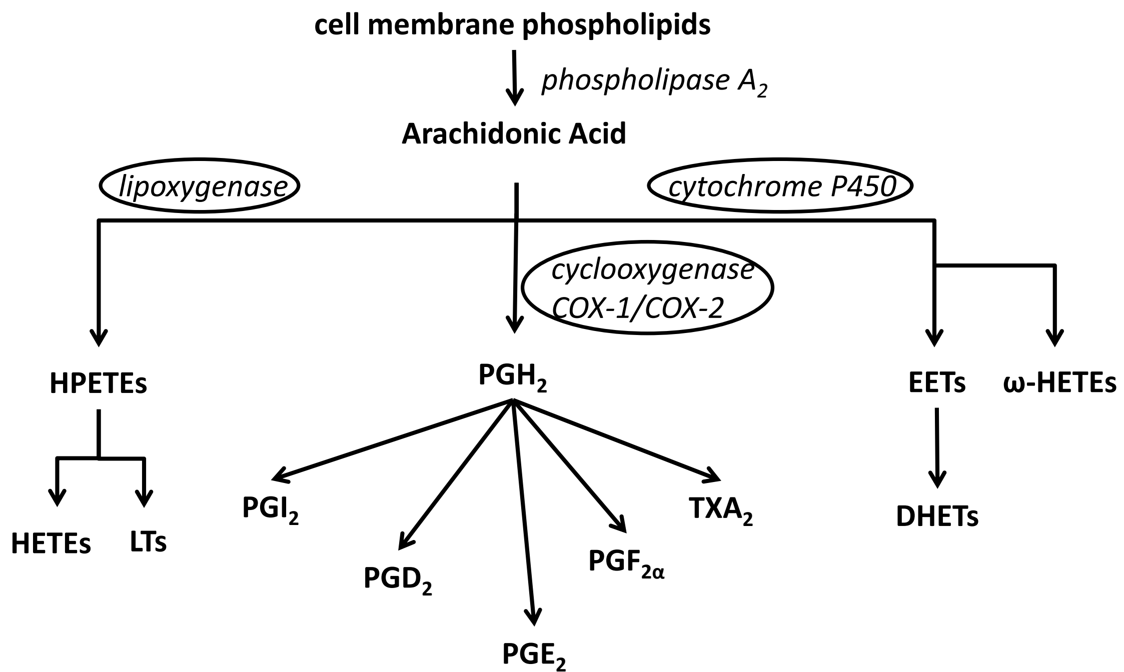

Figure 2. The three pathways of arachidonic acid metabolism.

1.1.2.2.1. Cyclooxygenase pathway

The two main isoforms of COX (COX-1/COX-2) are expressed constitutively in endothelial and vascular smooth muscle cells (20 fold higher expression in endothelium), and are both inducible by physical and/or humoral stimuli, such as shear stress (Doroudi *et al.*, 2000; Topper *et al.*, 1996). Recently a third COX enzyme, COX-3, was discovered. It is encoded by the same gene as COX-1 but retains an intron and thus is not functional in humans (Botting, 2003).

COX-1 is the better known constitutive form of the enzyme, as it is found in many tissues, including the vascular endothelium and platelets (Wallace, 1999). It is a housekeeping gene involved in the maintenance of physiological homeostasis (Smith *et al.*, 1996) . Because of the comparatively low levels of COX-2 expression, it was previously thought to be only inducible. However, recent studies have shown that it has an important regulatory role in the control of tissue function under various physiological states and is constitutively expressed in the heart, brain, kidneys, spinal cord, vasculature, and lung (Li *et al.*, 2005; Parente *et al.*, 2003; Wallace, 1999). Prostaglandins act on a wide array of cells and exert a variety of effects including modulation/regulation of vascular smooth muscle cells, platelet aggregation, inflammatory processes, calcium movement, cell growth, thermoregulation, glomerular filtration rate, stomach acid secretion, and induction of labor.

There are six major COX derived prostanoids produced in the endothelium: prostaglandin H_2 (PGH_2), prostaglandin I_2 (PGI_2, prostacyclin), prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin D_2 (PGD_2), and thromboxane A_2 (TXA_2) (Nakahata, 2008; Weeks, 1972). Although not definitive, in most tissues PGI_2, and PGD_2 act as vasodilators (Moncada *et al.*, 1979), PGH_2, $PGF_{2\alpha}$, and TXA_2 act as vasoconstrictors (Gluais *et al.*, 2005; Gluais *et al.*, 2006), and PGE_2 can act as either a dilator or constrictor, depending on tissue type, concentration, and receptor subtypes present (Carter *et al.*, 1986; Gluais *et al.*, 2005; Weeks, 1972). Prostaglandins are synthesized in almost all tissues, and the dominant PG synthesized in each tissue varies

with the enzymes that predominate. For example, PGI_2 is the most common PG in the vascular endothelium, while TXA_2 is the primary PG in the platelet (Chan *et al.*, 1986). These PGs are local mediators, acting in both autocrine and paracrine fashions, at or near their site of synthesis to trigger a vast array of biological signals, including vasodilation, vasoconstriction, and platelet aggregation (Moncada *et al.*, 1979; Nakahata, 2008; Parente *et al.*, 2003; Wallace, 1999; Weeks, 1972).

1.1.2.2.1.1. Prostaglandin H_2 (PGH_2)

Both COX-1 and COX-2 convert AA to PGH_2 in the cytosol. PGH_2, the common prostaglandin endoperoxide precursor of all PGs, is then further transformed by specific terminal synthases: thromboxane synthase (TXS) for TXA_2, prostacyclin synthase (PGIS) for PGI_2, and prostaglandin D-isomerase, prostaglandin E-isomerase, and prostaglandin F-reductase for PGD_2, PGE_2, and $PGF_{2\alpha}$, respectively. Importantly, in addition to its role as a common PG precursor, untransformed PGH_2 can interact at the TXA_2 receptor (TP) to induce vasoconstriction and platelet aggregation (Dai *et al.*, 1992; Feletou *et al.*, 2009; Hynes *et al.*, 1987; Lin *et al.*, 1994; Pagano *et al.*, 1991).

1.1.2.2.1.2. Prostaglandin I_2/Prostacyclin (PGI_2)

PGI_2 is synthesized by its terminal enzyme, PGI synthase (PGIS), in the vascular endothelium and vascular smooth muscle (Flavahan, 2007). The endothelium is the site for the majority of PGI_2 biosynthesis. PGIS is expressed in the vascular endothelium at much higher levels (5-100 fold) than any other terminal PG synthase, and as a result,

PGI_2 is the most abundantly produced prostanoid in the vasculature, with levels 10-100 fold higher than other PGs (Feletou *et al.*, 2009; Gluais *et al.*, 2005; Gluais *et al.*, 2006; Heymes *et al.*, 2000; Wu *et al.*, 2005). PGI_2 interacts with the prostacyclin receptor (IP) located on smooth muscle cells and platelets, activating vascular relaxation and inhibiting platelet aggregation via increases in cAMP level (Coleman *et al.*, 1994; Moncada *et al.*, 1979; Needleman *et al.*, 1986). Normally, PGI_2 is a potent vasodilator and a powerful endogenous inhibitor of platelet aggregation. At very high concentrations, however, PGI_2 interacts with the TXA_2 receptor (TP); this cross-activation induces vasoconstriction (Gluais *et al.*, 2006; Nakahata, 2008).

1.1.2.2.1.3. Thromboxane (TXA_2)

TXA_2 has opposing effects to PGI_2, it is a very potent vasoconstrictor (even more so than angiotensin II) and also induces platelet aggregation (Nakahata, 2008). It causes platelets to change shape and aggregate together promoting thrombus formation and thrombosis (Ally *et al.*, 1980). It was once thought to be mainly produced by platelets, but has been shown in more recent studies to also be produced (at much lower levels than PGI_2) in the endothelium and vascular smooth muscle by thromboxane synthase (TXS). The biosynthesis of TXA_2 is much lower in these tissues due to the lower expression of TXS in comparison to PGIS. TXA_2 acts at the TP receptor as the most potent agonist; however, other PGs also interact with TP with much lower affinities to produce vasoconstriction (Gluais *et al.*, 2005; Gluais *et al.*, 2006).

1.1.2.2.1.4. Other PGs: PGE_2, $PGF_{2\alpha}$, and PGD_2

PGE_2 has diverse effects depending on tissue type and which receptor subtype is expressed. PGE_2 is the most abundant prostaglandin in the human body, yet due to the limited expression of its terminal synthase in vascular smooth muscle, the contribution of PGE_2 in endothelium-dependent vascular function is much less than PGI_2 or TXA_2 (Tang *et al.*, 2008). There are four prostaglandin E_2 receptors (EP) (Sugimoto *et al.*, 2007), two of which cause vasodilation (EP_2, EP_4) via increases in cyclic adenosine monophosphate (cAMP), while the other two (EP_1, EP_3) cause vasoconstriction via increases in calcium mobilization/release and inhibition of cAMP (Alfranca *et al.*, 2006; Coleman *et al.*, 1994).

$PGF_{2\alpha}$ levels in the endothelium are much lower than PGI_2 due to the low levels of its terminal synthase PGFS (Gluais *et al.*, 2005; Gluais *et al.*, 2006; Tang *et al.*, 2008). $PGF_{2\alpha}$ interacts with its receptor (FP) expressed in the endothelium and vascular smooth muscle cells (Lake *et al.*, 1994) and can also interact with the TP receptor (Gluais *et al.*, 2005) to cause vasoconstriction.

The levels of PGD_2 tend to be very low in most vascular beds due to the very low levels of its terminal synthase PGDS. PGD_2 has similar albeit less potent actions to PGI_2. It inhibits platelet aggregation, increases cAMP levels and causes peripheral vasorelaxation (Walch *et al.*, 1999; Whittle *et al.*, 1978). Unlike PGI_2, PGD_2 can also

cause pulmonary vasoconstriction and bronchoconstriction (Johnston *et al.*, 1995; Wasserman *et al.*, 1980).

1.1.2.2.2 Lipoxygenase pathway

The second pathway of AA metabolism is via the lipoxygenase (LO) pathway. AA is metabolized by various oxidation reactions by several specific lipoxygenases (5,8,9,11,12, or 15-lipoxygenase) to hydroperoxyeicosatetraenoic acid (HPETE). Next HPETEs spontaneously hydrolyse to hydroxyeicosatetraenoic acids (HETEs) or leukotrienes (LT) (Borgeat *et al.*, 1979; Holtzman, 1991). For example the 5-LO pathway converts AA to 5-HPETE which is spontaneously hydrolysesd to 5-HETE or LTA_2. In humans, the role of the 5-LO pathway in bronchoconstriction and inflammation has been well studied. Current medications for asthma include 5-LO inhibitors and leukotriene receptor antagonists (Drazen, 1999).

1.1.2.2.3. Cytochrome P450 pathway

The third pathway of AA metabolism is via the cytochrome P450 (CYP) pathway. The major products of CYP-catalyzed AA metabolism are epoxyeicosatrienoic acids (EETs) and ω-hydroxyeicosatrienoic acid (HETEs) (Capdevila *et al.*, 2000). Metabolism of AA by CYP2J and CYP2C produces EETs (Zeldin, 2001). Soluble epoxide hydrolase (sEH) then catalyzes the hydrolysis of EETs to dihydroxyeicosatrienoic acids (DHETs) (Zeldin, 2001). In vascular smooth muscle cells, 20-HETE is the major product of CYP-catalyzed AA metabolism of CYP4A and CYP4F (Capdevila *et al.*, 2000). Eicosanoids

are produced in a cell and tissue specific manner. They exert effects on many biological functions as secondary messengers including regulation of vascular tone, ion transport, blood pressure, and control of cellular proliferation, inflammation and hemostasis (Maier *et al.*, 2001; Roman *et al.*, 2000; Zeldin, 2001). EETs and DHETs are potent vasodilators, while, 20-HETE is a potent vasoconstrictor. CYP-derived eicosanoids actions are generally mediated via large conductance Ca^{2+}-activated K^+ channels (BK_{Ca}). EETs have potent effects on vascular tone in numerous tissue beds including: coronary, cerebral, mesenteric, renal, pulmonary, and peripheral circulations (Kroetz *et al.*, 2002; Lu *et al.*, 2001; Medhora *et al.*, 2001; Zhang *et al.*, 2001). In the coronary circulation EETs have been identified as a putative EDHF, relaxing vascular smooth muscle cells by enhancing the opening of BK_{Ca} channels (Fisslthaler *et al.*, 1999; Pratt *et al.*, 2001). In addition to their actions as an EDHF-vasodilator, EETs also are potent anti-inflammatory agents, inhibit leukocyte adhesion to the vascular cell wall and protect against hypoxia-reoxygenation injury (Node *et al.*, 1999; Zeldin *et al.*, 2000).

In summary, healthy endothelial cells respond to a number of stimuli by releasing nitric oxide, which, in turn, stimulates vasorelaxation, inhibits platelet aggregation and reduces adhesion of leukocytes. In addition to nitric oxide, the products of AA metabolism, especially via the COX and CYP pathways, play important roles in modulating vascular tone. PGs act as either vasodilators (PGI_2) or vasoconstrictors (PGH_2, TXA_2), enhance or inhibit coagulation, and aid in the regulation of vascular tone and homeostasis. CYP

metabolites also play important roles in maintaining vascular homeostasis. EETs (and DHETs) cause vasodilation while 20-HETE has vasoconstrictor actions.

1.1.2.3. Endothelium derived hyperpolarizing factor (EDHF)

When nitric oxide and PGI_2 are inhibited, endothelium-mediated vasorelaxation can persist due a third mediator, endothelium derived hyperpolarizing factor (EDHF) (Urakami-Harasawa *et al.*, 1997). EDHFs cause hyperpolarization of the vascular smooth muscle cells and subsequently vasorelaxation. Although EDHF-mediated vasorelaxation has been reported in numerous studies, its chemical identity has not yet been fully elucidated. This is in part due to its variable nature and mechanisms of action depending on the species and tissue bed studied. However, EDHFs are known to play an important role in endothelium-dependent relaxation in both healthy and diseased states (Bauersachs *et al.*, 1996; Nishikawa *et al.*, 2000; Park *et al.*, 2008). Additionally, their contribution to relaxation increases as vessel size decreases, and thus they are important in the regulation of organ blood flow, peripheral vascular resistance and blood pressure, particularly when production of nitric oxide is compromised. Multiple EDHFs have been proposed, of which substantial evidence points to two main candidates, EETs, a cytochrome P-450-dependent metabolite of arachidonic acid (Archer *et al.*, 2003; Bellien *et al.*, 2006; Hecker *et al.*, 1994), as well as the reactive oxygen species, hydrogen peroxide (H_2O_2) (Yada *et al.*, 2003). Large-conductance, calcium-dependent K^+ (BK_{Ca}) channels are a common downstream effector for these potential EDHFs (Archer *et al.*, 2003; Liu *et al.*, 2011).

1.1.3. Endothelial dysfunction

Endothelial dysfunction, the loss of normal endothelial regulation of vascular structure and function, is exhibited in many disease states including coronary heart disease, hypertension, diabetes, and is also associated with advancing age. Endothelial dysfunction occurs when endothelial cells do not function normally. This is commonly characterized by a loss of the ability of the endothelial cells to release nitric oxide, especially if this loss is coupled with an enhancement in the release of constrictor prostanoids. This is the first step in a series of events leading to atherosclerosis and numerous cardiovascular diseases. Endothelial dysfunction alters vascular health in multiple ways including decreased nitric oxide bioactivity, increased superoxide production, attenuation of endothelium-dependent dilation, increased vascular tone and atherogenesis (Feletou *et al.*, 2006; Lyons, 1997; Vanhoutte, 1997; Vanhoutte, 1998; Vanhoutte *et al.*, 2009).

1.1.3.1. Alterations in endothelial-derived vasoactive mediators with disease

1.1.3.1.1. Coronary artery disease

Endothelial dysfunction is a key characteristic of cardiovascular disease (CVD). Its presence predicts the severity of outcome, particularly the occurrence of myocardial infarction and stroke (Suwaidi *et al.*, 2000; Vanhoutte, 1997). In patients with coronary artery disease, treatment with aspirin (COX inhibitor) and a TP receptor antagonist results in improved endothelial function (Belhassen *et al.*, 2003; Husain *et al.*, 1998). Thus, suggesting that endothelium-derived prostanoids contribute to endothelial

dysfunction in patients with coronary artery disease. Additionally, decreased synthesis or increased degradation leads to a decrease in the bioavailability of nitric oxide, which is a key manifestation of endothelial dysfunction that contributes to the pathogenesis of atherosclerosis (Vita, 2011).

1.1.3.1.2. Aging

Endothelial dysfunction increases in men after age 40 and in women after age 55 (Celermajer *et al.*, 1994). While the exact causes of age-dependent decreases in endothelial function are unknown, aging is usually associated with a reduction in the ability of the endothelium to elicit endothelium-dependent vasodilation in both animals and humans (Csiszar *et al.*, 2002; Taddei *et al.*, 2001; Tschudi *et al.*, 1996). This loss of function occurs via numerous mechanisms related to nitric oxide-mediated dilation including: increased activity of arginase, augmented production of oxygen derived free radicals, reduced expression of eNOS, lower eNOS activity, reduced expression of soluble guanylyl cyclase, and decreased nitric oxide release (Csiszar *et al.*, 2002; Kloss *et al.*, 2000; Taddei *et al.*, 2001; Tschudi *et al.*, 1996). In addition, an enhancement of vasoconstrictor prostanoids potentiates age-dependent endothelial dysfunction. With advancing age, both COX-1 and COX-2 expression are upregulated by oxidative stress. In addition, PGI_2 receptor (IP) expression decreases with age (Ge *et al.*, 1995; Numaguchi *et al.*, 1999; Shi *et al.*, 2008; Tang *et al.*, 2008). There is indirect evidence suggesting that untransformed PGH_2 is also augmented with aging due to COX-1 and/or COX-2 upregulation (Dai *et al.*, 1992; Hynes *et al.*, 1987; Lin *et al.*, 1994; Pagano *et al.*,

1991). Numerous studies have shown increases in TXA_2 and TXS mRNA in aorta and mesenteric arteries with age (Matz *et al.*, 2000; Tang *et al.*, 2008). Thus, the balance of dilator to constrictor prostanoids is altered with age, involving decreased sensitivity to PGI_2 and enhanced production of constrictor prostanoids PGH_2 and TXA_2, which eventually leads to endothelial dysfunction.

1.2. Therapeutic interventions

Numerous therapeutic interventions, including antioxidants, lipid-lowering drugs, exercise, and hormone replacement therapy, serve to improve both coronary and peripheral endothelial function. Some interventions aim to improve endothelial dysfunction by targeting one or more of the numerous risk factors that can cause endothelial damage: hypertension (angiotensin-converting enzyme inhibitors and calcium antagonists), hypercholesterolemia (lipid-lowering agents), cigarette smoking (cessation), sedentary lifestyle (increased physical activity), menopause (estrogen replacement therapy), and diabetes mellitus (control of associated metabolic abnormalities). Yet others provide beneficial changes to the endothelium by promoting vasorelaxation, inhibiting vasoconstriction, and reducing the production of free radicals (Cooke, 1997).

1.3. Dissertation scope

The endothelium plays an important regulatory role in maintaining vascular homeostasis. The progression from a healthy functional endothelium to a dysfunctional endothelium

underlies the development of numerous cardiovascular diseases. The restoration of endothelial function to a healthy state using therapeutic interventions is a major area of clinical interest. ***Thus, the goal of this dissertation research was to investigate the role of two therapeutic interventions (exercise training and hormone replacement therapy) on two states of endothelial dysfunction (chronic coronary occlusion and aging).***

2. PROJECT RATIONALE

2.1. Project 1- chronic coronary occlusion/exercise training

The first project utilized a model of chronic coronary artery occlusion to evaluate the effects of exercise training on cellular and molecular adaptations of the nonoccluded control and collateral-dependent vasculature.

2.1.1. Coronary artery disease

2.1.1.1. Epidemiology

Coronary artery disease is not only the most common type of heart disease, it is also one of the most common causes of death worldwide claiming over 400,000 lives in 2009 (Go *et al.*, 2013). That same year, coronary artery disease cost over $195 billion dollars in direct health care services, medications, and lost productivity. It is estimated that one-half of all middle aged men and one-third of all middle aged women in the United States will develop some manifestations of coronary artery disease (Lloyd-Jones *et al.*, 1999). Cardiovascular diseases are the major cause of death and disability in the United States in every major ethnic group for both males and females, causing a higher mortality rate each year than the next four leading causes combined: cancer, chronic lower respiratory diseases, accidents and diabetes mellitus (Lloyd-Jones *et al.*, 2010; Rosamond *et al.*, 2008). While the death rate for coronary vascular disease has continued to decline over the last decade, the burden of the disease remains high. This year alone, an estimated 635,000 Americans will have a new coronary attack, 280,000 will have a recurrent

attack, and an additional 150,000 are estimated to have silent myocardial infarctions. By 2030, 40.8% of Americans are projected to have some form of coronary vascular disease (Go *et al.*, 2013).

2.1.1.2. Hemodynamic significance of coronary artery stenosis on myocardial function

Coronary artery disease is characterized by atherosclerosis of the coronary arteries, the hallmark of which is atherosclerotic plaques, which progressively narrow the coronary artery lumen and impair myocardial blood flow (Falk, 1982). Depending on the severity of the obstruction and the speed of development, this reduction of blood flow may be symptomatic or asymptomatic, occur at rest or during exertion, and result in a myocardial infarction. It is difficult to determine the hemodynamic significance of coronary artery stenosis because severe stenotic lesions may be bypassed and adequately compensated for by collateral vessels (Falk, 1982).

Animal models have provided key information about the relationship between the degree of stenosis and its hemodynamic significance. Critical stenosis is the degree of obstruction at which the peripheral vascular bed is maximally dilated and any further increase in lesion size will cause significant decreases in blood flow and myocardial ischemia at rest. Numerous studies have shown that blood flow distal to stenosis remains normal until luminal area is extremely reduced with severe stenosis (Furuse *et al.*, 1975; Gould *et al.*, 1975; Gould *et al.*, 1974). Blood flow is maintained by autoregulatory

vasodilation of the vascular bed distal to occlusion. This peripheral vasodilation increases the pressure drop across stenosis by lowering arterial pressure distal to the lesion and maintains flow at normal or near-normal levels (Gould *et al.*, 1975; Roth, 1976). Abundant studies in canine preparations have reported that resting myocardial blood flow fails to meet demand when an isolated proximal coronary stenosis reduces the luminal cross-sectional area by 80-95% (Furuse *et al.*, 1975; Gould *et al.*, 1975; Gould *et al.*, 1974; Roth, 1976).

2.1.1.3. Chronic coronary occlusion as a model of coronary artery disease

The coronary collateral circulation supplies blood flow to the compromised myocardial regions distal to complete coronary artery occlusion. During progressive arterial occlusion, blood flow to the compromised myocardial region downstream of the stenosis is maintained via angiogenesis (growth of new collateral vessels) and arteriogenesis (enlargement of preexisting collateral vessels) (Buschmann *et al.*, 2000). In humans this sparse collateral vessel network is often sufficient to prevent ischemia and maintain myocardial function at rest, however myocardial function remains compromised during myocardial stress such as exercise (Kolibash *et al.*, 1982).

It is important to choose the most relevant animal models for human ischemic heart disease. For many years the dog was used to study chronic coronary occlusion; however, their innate collateral circulation is very substantial and capable of providing up to 40% of normal blood flow to the perfusion bed during acute occlusion (Hearse, 2000;

Maxwell *et al.*, 1987). Conversely, acute occlusion in pigs normally results in infarct of the entire compromised region due to the sparse native collateral circulation (Savage *et al.*, 1981). Porcine and human coronary anatomy and physiology are very similar, including minimal innate coronary collateral vessels, a right-dominant coronary system and analogous cardiac conduction systems (Hearse, 2000; Maxwell *et al.*, 1987). Consequently, pigs are an excellent model of collateral vascular development in response to myocardial ischemia in humans.

When coronary artery occlusion progresses gradually, sufficient collateral vessel recruitment and growth can occur, and as a consequence when complete stenosis is reached there is little or no infarction of the dependent myocardium. Collateral vessels are able to provide adequate arterial flow to maintain myocardial integrity during resting conditions, but the ability to augment blood flow in response to exercise or other stress may be limited. The ameroid constrictor technique is commonly used to induce collateral vessel growth via slow progressive occlusion. An ameroid constrictor consisting of an inner ring of casein surrounded by a stainless steel sheath is surgically implanted around a coronary artery. Casein, a hygroscopic substance, slowly swells as it absorbs body fluid. Gradual occlusion occurs as the stainless steel sheath forces the casein to swell inwardly, progressively compressing the artery until complete occlusion occurs around 14-30 days (Elzinga, 1969).

2.1.2. Exercise therapy

2.1.2.1. Effect of exercise training on endothelial function in coronary artery disease patients

The effect of exercise on the vascular health of patients with coronary artery disease is of considerable interest and the benefits of regular exercise following a cardiac event are quite significant. Exercise-based cardiac rehabilitation for patients with coronary artery disease decreases total mortality by 20% and cardiac mortality by 26% (Taylor *et al.*, 2004). Participation in a comprehensive cardiac exercise based rehabilitation program leads to a significant reduction in cardiac events and hospital readmissions and a significant increase in functional capacity (Ades *et al.*, 1997; Hedback *et al.*, 2001).

The effect of exercise training on endothelial function in patients with coronary artery disease has been examined in numerous clinical studies. After a 12 month exercise regimen, exercise-trained patients showed significant improvement in myocardial perfusion of the ischemic region as compared to sedentary patients (Kendziorra *et al.*, 2005). In another study exercise-training (4 weeks, stationary bike) reduced acetylcholine-induced coronary artery constriction, enhanced endothelial nitric oxide release, and increased coronary flow reserve (Hambrecht *et al.*, 2000). In a follow-up study, patients with stable coronary artery disease that were to undergo elective bypass surgery were assigned to a 4 week exercise training or sedentary protocol. In agreement with previous findings, exercise training improved agonist-mediated endothelium-dependent vasodilatory capacity, and enhanced both phosphorylated (Ser1177) and total

eNOS protein levels in left internal mammary artery of patients with stable coronary artery disease (Hambrecht *et al.*, 2003). A large scale clinical trial of more than 100 patients with stable coronary artery disease compared the effects of exercise training to the effects of standard percutaneous coronary intervention (PCI) with stenting. Exercise training was associated with a higher event-free survival, increased maximal oxygen uptake, and lower overall treatment cost (Hambrecht *et al.*, 2004).

In summary, this series of studies in patients with coronary artery disease provides significant clinical evidence that exercise training improves endothelial function and myocardial perfusion. However, the mechanisms underlying the enhanced coronary/collateral vasomotor responsiveness in improving myocardial function and perfusion with exercise training in coronary artery disease patients have not been fully elucidated.

2.1.2.2. Effect of exercise training on blood flow to collateral-dependent myocardium

As previously stated, there are many similarities between porcine and human coronary circulation in terms of collateral development and persistent regional myocardial dysfunction under conditions of increased myocardial oxygen demand such as with exercise. Thus, the porcine model has been used extensively to examine adaptations in the collateral circulation in response to chronic coronary occlusion and exercise training. Bloor and colleagues conducted a series of experiments to characterize the adaptations in

collateral development in response to myocardial ischemia and exercise-training (Bloor *et al.*, 1984; Roth *et al.*, 1990). In the first study, radiolabeled microspheres were used to determine blood flow into collateral-dependent and nonoccluded myocardial regions (Bloor *et al.*, 1984). Flow in the proximal left circumflex (above the occlusion) did not differ from the nonoccluded left anterior descending artery. Chronic occlusion stimulated collateral development in and around the compromised ischemic region. Additionally, blood flow distal to the occlusion (in the collateral-dependent region) was significantly increased after 5 months of exercise-training indicating enhanced collateral development with exercise (Bloor *et al.*, 1984). In a follow-up study, the effects of exercise-training on myocardial function after chronic coronary occlusion were examined at rest and during moderate and severe exercise (Roth *et al.*, 1990). In the sedentary group, blood flow ratios (flow in the collateral-dependent region as compared to flow in the normally perfused region) were significantly increased in the endocardium during moderate exercise. In the exercise-trained group, blood flow ratios were even further enhanced in the endocardium during moderate exercise, and were also significantly increased in the endocardium, midmyocardium and epicardium during severe exercise. Interestingly, regional myocardial function improved in a corresponding manner. Exercise training improved systolic wall thickening in the collateral-dependent region at both moderate and severe exercise levels as compared to the pre-exercise stress test. Sedentary animals also showed improvements in systolic wall thickening at moderate exercise levels, although these improvements were not as prominent as seen in the exercise-training group (Roth *et al.*, 1990). In additional studies, exercise training

improved myocardial function, increased coronary collateral reserve in the collateral-dependent region, and enlarged total cross-sectional area of the vascular bed (White *et al.*, 1998; White *et al.*, 1992). Taken together, these studies suggest that exercise training enhances collateral development and subsequently improves myocardial function of the collateral-dependent myocardial region in the presence of critical stenosis or complete occlusion.

2.1.2.3. Effect of exercise training on coronary vascular reactivity in the collateral-dependent myocardium

Exercise training improves endothelium-dependent relaxation (Griffin *et al.*, 1999) and adenosine-induced relaxation (Heaps *et al.*, 2000) in epicardial arteries within the collateral-dependent region. Additional studies found that exercise training restored endothelium-dependent vasodilation in response to bradykinin in porcine arterioles isolated from collateral-dependent myocardium (Griffin *et al.*, 2001). Furthermore, vasodilation produced by vascular endothelial growth factor ($VEGF_{165}$) in these vessels was enhanced by exercise training, and mediated primarily by increased nitric oxide bioavailability (Fogarty *et al.*, 2004). Basal myogenic tone in both collateral-dependent arterioles is enhanced with exercise, similar to the effect of exercise training on arterioles in normal hearts (Muller *et al.*, 1993). This increase in basal tone is associated with augmented vasodilator influences exerted by increased nitric oxide production and K_V channel activity (Heaps *et al.*, 2006).

2.1.2.4. Effect of exercise training on nitric oxide

Exercise training improves endothelial function of both normal and diseased arteries. Moderate exercise training has a beneficial effect on collateral-dependent myocardial perfusion in human studies (Belardinelli *et al.*, 1998; Hambrecht *et al.*, 2003; Hambrecht *et al.*, 2000). Similarly, exercise training restores endothelium-dependent vasodilation in porcine arterioles isolated from collateral-dependent myocardium (Griffin *et al.*, 1999; Roth *et al.*, 1990; Xie *et al.*, 2013). Exercise training enhances endothelium-dependent relaxation, endothelial nitric oxide synthase (eNOS) mRNA expression, and eNOS protein levels in coronary arteries and arterioles of control animals (Laughlin *et al.*, 2001; Sessa *et al.*, 1994). Exercise training increases nitric oxide generation, eNOS and p-eNOS gene expression in animal models of disease (Graham *et al.*, 2004; Grijalva *et al.*, 2008; Heaps *et al.*, 2006; Tanabe *et al.*, 2003; Zhou *et al.*, 2010) and in human patients with coronary artery disease (Hambrecht *et al.*, 2003; Hambrecht *et al.*, 2000). However, the cellular and molecular mechanisms responsible for improvement in nitric oxide bioavailability in coronary artery disease subsequent to exercise therapy have not been fully elucidated.

A series of studies by Heaps/Parker and colleagues using the porcine ameroid occluder model of chronic coronary occlusion have examined the effect of exercise training on endothelial-mediated relaxation and the contribution of nitric oxide. Endothelium-dependent relaxation to bradykinin and ADP was enhanced with exercise training (Griffin *et al.*, 1999). These functional endothelial improvements were partially

attributed to increased contribution of nitric oxide with exercise training. Furthermore, exercise training significantly enhanced bradykinin-mediated increases in endothelial calcium levels, nitric oxide levels, and the distribution of eNOS/caveolin-1 ratio at the plasma membrane in endothelial cells of nonoccluded control and collateral-dependent arteries (Zhou *et al.*, 2010). Additionally, total eNOS and phosphorylated eNOS (pSer1179) levels were increased significantly with exercise training. Taken together, these findings provide insight into exercise training-induced adaptations in cellular mechanisms of nitric oxide regulation that contribute to enhanced nitric oxide production and agonist-mediated relaxation in arteries of occluded/stenosed hearts (Griffin *et al.*, 1999; Griffin *et al.*, 2001; Heaps *et al.*, 2006; Zhou *et al.*, 2010).

2.1.2.5. Effect of exercise training on other vascular mediators

Under normal conditions, the major relaxing factors released from the endothelium include nitric oxide, prostacyclin and EDHF. As discussed previously in detail, the relationship between physical exercise and nitric oxide has been well studied. In contrast to the abundant literature on the role of nitric oxide in the vascular adaptations to exercise, far less is known regarding the changes in other vascular mediators during physical exercise (Figure 3).

A few studies have examined the role of PGI_2 in blood flow and vascular reactivity in collateral-dependent vasculature. PGI_2 is by far the most abundant prostaglandin generated in vascular endothelial cells and is a potent vasodilator of coronary arteries

and resistance vessels. In dogs with chronic coronary occlusion, prostaglandins significantly contribute to blood flow in the collateral-dependent region but not in the normally perfused myocardium (Altman *et al.*, 1992). In agreement, enhanced contribution of vasodilator prostanoids has been reported distal to chronic coronary occlusion (Rapps *et al.*, 1997). In patients with atherosclerotic coronary artery disease undergoing diagnostic cardiac catheterization, indomethacin significantly decreased coronary sinus blood flow and increased myocardial oxygen extraction (Duffy *et al.*, 1999; Friedman *et al.*, 1981; Pacold *et al.*, 1986). This vasoconstriction in response to cyclooxygenase blockade appears to be facilitated by the presence of disease, since there was no significant change in coronary hemodynamics in response to cyclooxygenase blockade with ketoprofen in human subjects with angiographically normal coronary arteries (Neri Serneri *et al.*, 1990b), yet inhibition of prostanoids significantly enhanced coronary vascular resistance in patients with coronary artery disease (Neri Serneri *et al.*, 1990a). Taken together, these data show that, PGI_2 does not appear to influence coronary blood flow during basal conditions in normal human subjects but may exert a vasodilator influence in patients with atherosclerotic coronary artery disease. The effect of exercise training on cyclooxygenase contribution to blood flow to compromised myocardium in humans or animals has not yet been fully elucidated.

Several studies have suggested that a cross-talk interaction between nitric oxide and PGI_2 exists. Nitric oxide exerts an inhibitory effect on PGI_2 production in vitro (Osanai *et al.*, 2001; Osanai *et al.*, 2000). Interestingly, inhibition of prostanoids significantly

blunted the duration of reactive hyperemia in dogs chronically treated with LNAME but had no effect in control dogs (Puybasset *et al.*, 1996). Thus, the contribution of PGI_2 in mediating vasodilation appears to be augmented when nitric oxide bioavailability is low.

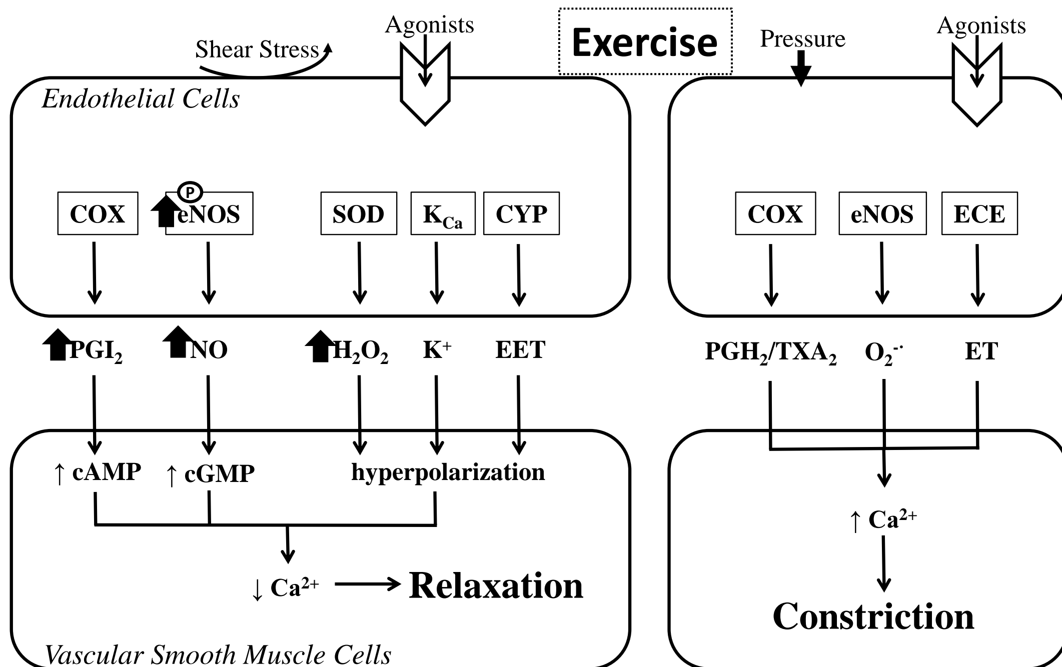

Figure 3. Alterations in the mechanisms involved in endothelium-dependent relaxation with exercise therapy.

2.1.3. Aims of Project 1

Exercise training improves nitric oxide-dependent dilation; however, the effects of exercise on other compensatory vasodilator pathways are unknown. Thus, it is important to determine to what extent the roles of other endothelial mediators besides nitric oxide, including PGI_2 and EDHFs, are altered by exercise training. ***The central hypothesis to be tested is that:***

Exercise training will increase the contribution of multiple compensatory pathways, including nitric oxide, PGI_2, and EDHFs, in the underlying setting of chronic coronary occlusion.

This hypothesis will be tested by addressing the following specific aims:

Specific Aim 1: Determine the effects of chronic coronary artery occlusion and exercise training on vascular endothelial function.

Specific Aim 2: Determine the effects of chronic coronary artery occlusion and exercise training on basal and agonist-stimulated production of nitric oxide and PGI_2.

Specific Aim 3: Determine the effects of chronic coronary artery occlusion and exercise training on whole cell K^+ and BK_{Ca} channel currents.

2.2. Project 2- aging/hormone replacement therapy

2.2.1. Aging

The second project utilized a model of aging to evaluate the interactive effects of age and hormone replacement therapy on cellular and molecular mechanisms underlying cerebrovascular function.

2.2.1.1. Epidemiology

Growth of the older population has increased remarkably over the last century. A decline in infant and child mortality has led to an increase in life expectancy from 47.3 years to 68.2 years over the first half of the twentieth century. Additionally, in the second half of the twentieth century, life expectancy continued to rise due to an increase in the survival of middle- and old-aged populations. This decline in mortality throughout the lifespan has resulted in a population with an increasingly large fraction of individuals surviving to old age. In 1900, 4.1% (of the 76 million people in the United States) were aged 65 and older. By 1950, this rate doubled to 8%, and further increased in 2000 such that 12.3% of the 276 million people in the United States were over the age of 65. Similar to younger individuals, heart disease is the leading cause of death in individuals aged 65 and older, followed by cancer, stroke, chronic lower respiratory tract diseases, and Alzheimer's disease. In addition, quality of life declines with advancing age, with increased onset of arthritis and chronic joint pain, dementia, loss of vision and hearing, depression, and increased physical limitations (Ferrucci *et al.*, 2008; Lutz *et al.*, 2008). The dramatic increase in the proportion of elderly in the United States' population has

wide-ranging impacts throughout society including increased demand for medical care and social services.

2.2.1.2. Age-related endothelial dysfunction

Vascular aging is associated with structural and functional changes in the extracellular matrix, endothelium, and vascular smooth muscle of blood vessels. Arterial stiffening occurs with advancing age due to increases in vessel thickness, vessel wall collagen content, and size or number of smooth muscle cells (Guyton *et al.*, 1983; Moreau *et al.*, 1998).

In the vascular endothelium, aging causes endothelial thickening and increases the presence of mononuclear cells (Guyton *et al.*, 1983). Functionally, advancing age results in a reduction in endothelium-dependent vasodilation (Csiszar *et al.*, 2002; Taddei *et al.*, 2001; Tschudi *et al.*, 1996). Age-related alterations in vascular reactivity are due to changes in the equilibrium between endothelium-derived relaxing and contracting factors. With advancing age there is a progressive decrease in the roles of nitric oxide and PGI_2 with an increase in oxygen-derived free radicals and COX-derived constrictor prostanoids. Decreases in nitric oxide-mediated dilation with age occur due to increased activity of arginase, augmented production of oxygen derived free radicals, reduced expression of eNOS, lesser eNOS activity, reduced expression of soluble guanylyl cyclase, and decreased nitric oxide release (Csiszar *et al.*, 2002; Kloss *et al.*, 2000; Taddei *et al.*, 2001; Tschudi *et al.*, 1996). In humans, aging is associated with a decrease

in urinary excretion of the stable metabolite of PGI_2, 6-keto-prostaglandin $F_{1\alpha}$ (Hornych *et al.*, 1991). In addition, PGI_2 receptor (IP) expression decreases with age (Numaguchi *et al.*, 1999; Tang *et al.*, 2008). In contrast, an enhancement of vasoconstrictor prostanoids and an increase in reactive oxygen species potentiates age-dependent endothelial dysfunction. With advancing age, both COX-1 and COX-2 expression are upregulated by oxidative stress (Ge *et al.*, 1995; Numaguchi *et al.*, 1999; Shi *et al.*, 2008; Tang *et al.*, 2008). Indirect evidence suggests that untransformed PGH_2 is also augmented with aging due to COX-1/COX-2 upregulation (Dai *et al.*, 1992; Hynes *et al.*, 1987; Lin *et al.*, 1994; Pagano *et al.*, 1991). Numerous studies have shown increases in TXA_2 and TXS mRNA in aorta and mesenteric arteries with age (Matz *et al.*, 2000; Tang *et al.*, 2008). Additionally, several studies have reported increases in the vascular formation of superoxide with aging (Blackwell *et al.*, 2004; Csiszar *et al.*, 2002). Thus, the balance of endothelial dilator to constrictor factors is altered with age. This involves decreased role of nitric oxide and PGI_2, and enhanced production of constrictor prostanoids PGH_2 and TXA_2; eventually leading to endothelial dysfunction (Figure 4).

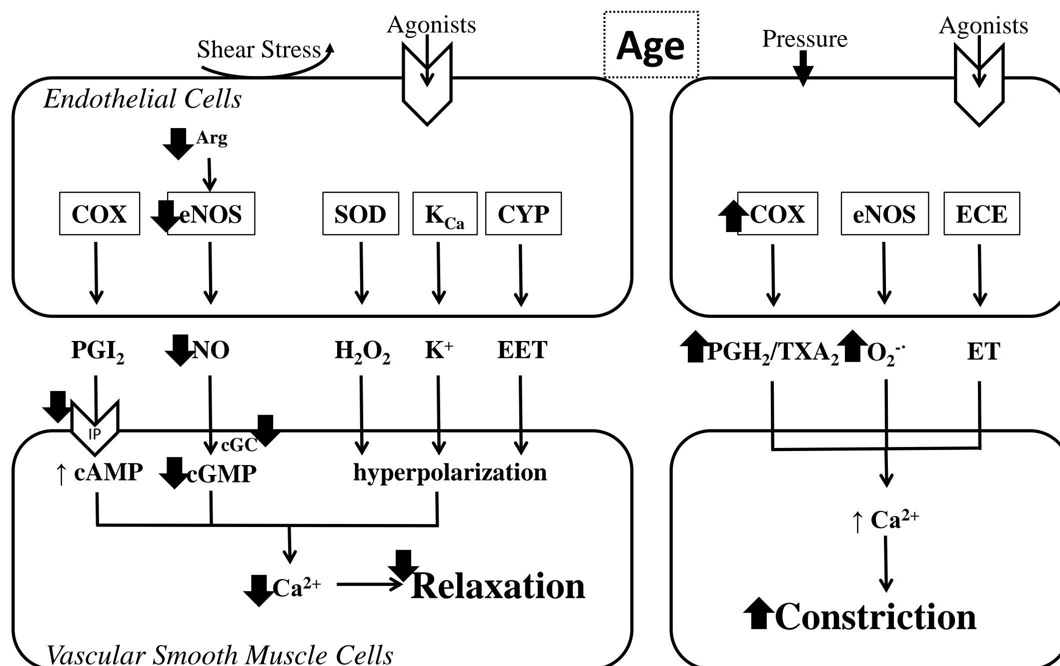

Figure 4. Alterations in the mechanisms involved in endothelium-dependent relaxation with advancing age.

2.2.2. Hormone replacement therapy

2.2.2.1. Synthesis of gonadal steroids

In both males and females, the gonads and adrenal glands synthesize and secrete estrogens and androgens into the general circulation. In females, after puberty, the ovaries synthesize much greater amounts of estrogen in a cyclic fashion to regulate ovulation and reproductive capacity. 17β-estradiol is the principal and most potent estrogen released in nonpregnant females; estrone and estriol are also present at much lower concentrations. In males, the testes synthesize and secrete high levels of testosterone which maintains the male sexual phenotype. The enzyme aromatase, found

in both the periphery and the brain, is responsible for the local conversion of circulating androgens to estrogens in both sexes. The greatest peripheral source of aromatase in both males and females is the adipose tissue. In the human brain, aromatase is found in the highest levels in the hypothalamus. It is found in significantly higher levels in males and is regulated by estradiol in both males and females. Significant but similar low levels of aromatase are found in both sexes in other regions of the brain including the amygdala, hippocampus, midbrain and cortical regions (Roselli *et al.*, 2009). Adding additional complexity, besides the synthesis of estradiol from circulating gonadal and adrenal precursors with aromatase, the brain also possesses all of the enzymes required for the de novo synthesis of steroids from cholesterol (Garcia-Segura, 2008).

2.2.2.2. Mechanisms of estrogen signaling in the brain

2.2.2.2.1. Estrogen receptors

The classic genomic effects of estrogen in the brain are mediated by nuclear estrogen receptors (ER). There are two known isoforms (ERα and ERβ) found widely throughout the brain with differing distributions. The binding of estrogen to its intracellular receptor causes receptor-ligand dimerization and interactions with other co-factors in the cell. This dimer complex can then bind directly to estrogen response elements (EREs) within the promoter region of specific genes to alter transcription rate (McEwen *et al.*, 1999).

ERα is widely expressed in brain regions involved in the control of reproduction including the hypothalamic, preoptic, and limbic structures (anteroventral periventricular

nucleus (AVPV), medial preoptic nucleus (MPN), median preoptic area, bed nucleus of the stria terminalis (BST), lateral septum, medial amygdala, arcuate nucleus (ARC), periventricular nucleus of the hypothalamus, and ventromedial nucleus (VMN)). Estrogen can also exert actions that are not regulated by the hypothalamus/preoptic regions including neuroprotection, locomotor activity, mood, memory, and cognition. Thus, it is also expressed in areas not traditionally associated with reproduction specifically the olfactory regions, cerebellum, area postrema and substantia gelatinosa of the spinal cord. ERβ is distributed in some limbic-hypothalamic regions where ERα is present (BST, medial preoptic area, MPN, medial amygdala, and AVPV), and is also expressed in areas without ERα (diagonal band of Broca, supraoptic area, and paraventricular nucleus). ERβ is also widely expressed in areas not associated with reproduction including the hippocampus, olfactory regions, spinal cord, cerebellum, substantia nigra, ventral tegmental area, dorsal Raphe and locus coeruleus (Laflamme *et al.*, 1998; Mitra *et al.*, 2003; Shughrue *et al.*, 1997; Simerly *et al.*, 1990). This broad distribution of estrogen in the brain allows estrogen to regulate diverse targets and exert a board range of actions. Each ER isoform regulates unique sets of target genes in tissue and cell-specific manners due to the unique distribution of ERα and ERβ in the brain and the formation of homo- or heterodimers. Although circulating estrogen can enter the brain, its effects are determined by which of the two ER isoforms is expressed (or co-expressed), the ratio of ERα to ERβ in a particular brain region, the presence of coregulator proteins, and the binding to EREs to enable gene transcription. Thus, the

actions of estrogen can be differentially transduced throughout the brain (Chakraborty *et al.*, 2004; Gillies *et al.*, 2010).

The role of aging in the regulation of ERs is not well understood. Interestingly, using receptor-specific knockout animals, ERα mediates the neuroprotective effects of estrogen following ischemic injury (Dubal *et al.*, 2001). Additionally, expression of ERα is altered by age. It is significantly increased in the olfactory bulb of older reproductively senescent females (Jezierski *et al.*, 2001), and in the AVPV and VMN regions of middle aged and old aged female rats (Chakraborty *et al.*, 2003). In humans, ERα expression is altered with age from a nuclear localization in young females to a more cytoplasmic localization in older females (Hestiantoro *et al.*, 2004). More research is necessary to provide a better understanding of age-related changes in ER expression and their regulation by gonadal hormones.

2.2.2.2.2. Membrane estrogen receptor signaling

Estrogens can also initiate rapid signaling (seconds to minutes) via interactions with the cell membrane in many brain regions. These actions probably involve a membrane ER that is not a transcription factor; however, the identity of this membrane receptor has not been fully elucidated thus far. Although these mechanisms are commonly referred to as nongenomic actions in order to distinguish them from the classic mode of steroid action, recent findings have shown that they may also act to regulate gene transcription. Emerging evidence reveals that classic "nuclear" ERα and ERβ receptors, and other

novel receptors such as G-protein coupled ER30 (GPR30), can be localized at the cell membrane to initiate rapid activation of intracellular signaling pathways (Mermelstein, 2009; Micevych *et al.*, 2009; Raz *et al.*, 2008; Revankar *et al.*, 2005; Vasudevan *et al.*, 2008). Estrogen directly stimulates Ca^{2+} channels and intracellular Ca^{2+} stores. This mobilization of Ca^{2+} activates pathways involving: Ca^{2+}-CAM-dependent kinases, cAMP-dependent kinases, mitogen-activated protein kinases (MAPK), extracellular signal-related kinases (ERK), and phosphoinositide 3-kinases (PI3K) (Boulware *et al.*, 2005; Mermelstein, 2009; Vasudevan *et al.*, 2008). Additionally estrogen-activated signaling pathways increase mitochondrial efficiency consequently leading to decreased free radical generation in the brain (Brinton, 2008; Chen *et al.*, 2009). It is interesting to note that most cellular mechanisms of estrogen have critical roles in cell survival, apoptosis, function, and neurodevelopment; and thus may promote the neuroregulatory, neurotrophic, and neuroprotective effects of estrogen in brain physiology and pathophysiology (Gillies *et al.*, 2010).

2.2.2.3. Effects of estrogen on cerebrovascular reactivity and blood flow

Cerebrovascular function including reactivity and subsequent changes in blood flow into a given region is affected by variations in circulating estrogen levels. Overall, estrogen appears to enhance cerebral blood flow (CBF). Women tend to exhibit higher levels of CBF than men when they are younger, but this difference becomes less significant later in life, around the onset of menopause (Rodriguez *et al.*, 1988; Shaw *et al.*, 1984). Additionally CBF varies throughout the menstrual cycle (Brackley *et al.*, 1999; Diomedi

et al., 2001) and is altered throughout pregnancy (Brackley *et al.*, 1998). Additionally, in postmenopausal women HRT also enhances CBF (Penotti *et al.*, 1993; Slopien *et al.*, 2003).

Chronic exposure to estrogen positively alters cerebrovascular reactivity by decreasing cerebral vascular tone and increasing cerebral blood flow by enhancing endothelial derived nitric oxide and prostacyclin pathways. Interestingly, there appear to be striking sex-differences in the modulation of cerebrovascular myogenic tone, with male arteries constricting more in response to increasing pressure compared to females (Geary *et al.*, 1998). Numerous studies have reported that estrogen enhances the production of and/or the sensitivity of cerebral arteries to vasodilatory factors (Geary *et al.*, 2000a; Geary *et al.*, 1998; Ospina *et al.*, 2003; Ospina *et al.*, 2002; Pelligrino *et al.*, 2000; Skarsgard *et al.*, 1997). These actions appear to be mediated primarily through changes in vascular endothelial mediators including nitric oxide, PGs and EDHF (Figure 5).

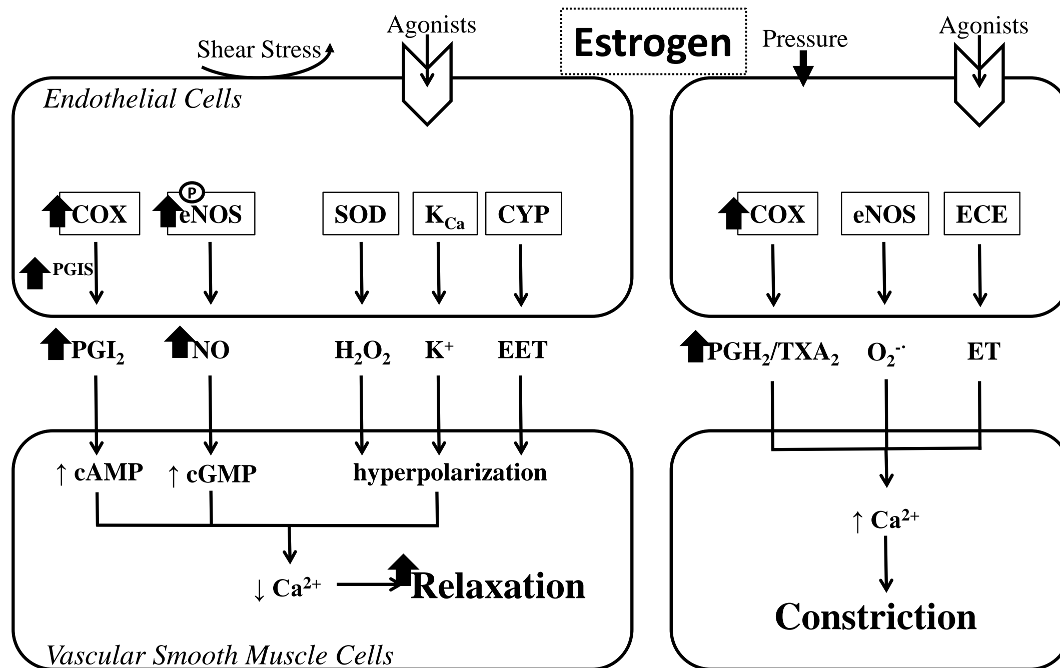

Figure 5. Alterations in the mechanisms involved in endothelium-dependent relaxation with estrogen.

2.2.2.4. Effect of estrogen on endothelial mediators

2.2.2.4.1. Nitric oxide

The ability of estrogen to enhance nitric oxide production via eNOS is perhaps the most well-studied effect of the sex steroids on the cerebrovasculature. Estrogen activates numerous mechanisms that act in concert to increase eNOS function. Classic genomic actions of estrogen are activation of ER receptor to stimulate eNOS gene expression, mRNA and protein. Estrogen also activates the PI3K signaling pathway subsequently phosphorylating eNOS at serine 1177/1179 which increases eNOS activity as well as its sensitivity to Ca^{2+} (Stirone *et al.*, 2005). Additionally, estrogen modulates other regulatory proteins of eNOS including caveolin-1 and CaM. Levels of the inhibitory

protein caveolin-1 are highest in pial arteries of ovariectomized rats as compared to intact or ovariectomized rats receiving estrogen treatment. Furthermore, these estrogen-mediated decreases in caveolin-1 positively correlate with increases in eNOS activity (Santizo *et al.*, 2002; Sobey *et al.*, 2004; Xu *et al.*, 2001). CaM, a regulatory protein necessary for Ca^{2+} activation of eNOS, is also enhanced by estrogen in cerebral arteries (Sobey *et al.*, 2004). Numerous clinical studies and animal experiments have shown that cerebral arteries from females and chronically estrogen-treated animals display greater nitric oxide-dependent dilation as compared to males or ovariectomized females (Geary *et al.*, 2000a; Geary *et al.*, 1998; Geary *et al.*, 2000b; Pelligrino *et al.*, 2000; Skarsgard *et al.*, 1997). Interestingly, other sex steroids do not appear to modulate nitric oxide in the cerebrovasculature. Neither testosterone nor progestins have any effect on eNOS levels or nitric oxide-mediated dilation. (Geary *et al.*, 2000b; McNeill *et al.*, 2002). Testosterone increases myogenic tone by endothelium-dependent mechanisms sensitive to COX and/or K^+ channel inhibition (Geary *et al.*, 2000b).

2.2.2.4.2. Prostaglandins

Estrogen enhances endothelium-dependent dilation that is dependent on NOS (as mentioned above) or COX. Estrogen shifts the balance of prostanoid synthesis towards greater production of vasodilator prostanoids, whereas testosterone favors production of constrictor prostanoids (Ospina *et al.*, 2003). In cerebral vessels, estrogen elevates both COX-1 and PGIS resulting in enhanced PGI_2 production (Geary *et al.*, 2000a; Ospina *et al.*, 2003; Ospina *et al.*, 2002). Interestingly, TXA_2 production was also slightly but

significantly elevated in young animals with estrogen-treatment, possibly a reflection of increased COX-1 levels (Lin *et al.*, 2002; Ospina *et al.*, 2002).

The effects of estrogen on the prostanoid pathway have been examined in a series of studies by Stallone and colleagues. Reactivity of the rat thoracic aorta to vasopressin is substantially greater in females than in males or ovariectomized females. Importantly, estrogen replacement therapy of ovariectomized females restored reactivity to VP to that of the intact female. Selective blockade of various prostanoid pathway enzymes and/or receptors revealed that constitutive forms of COX-2 and thromboxane synthase (TXS) exist in the systemic vasculature. Enhanced responsiveness to VP in females was attributed to enhanced production of the constrictor prostanoid thromboxane and increased expression of COX-2, TXS and the TP receptor (Li *et al.*, 2008; Li *et al.*, 2005; Sellers *et al.*, 2008).

2.2.2.5. Model of hormonal aging

There are very few animal models that properly replicate human menopause. Endocrine aging is a slow, continuous, multi-stage process in humans. In humans, the loss of ovarian follicles with menopause results in a decline in estrogen levels (Judd *et al.*, 1994). During perimenopause, menstrual cycles become irregular, estrogen levels decline and fluctuate, and follicle-stimulating hormone (FSH) levels rise. This eventually leads to menopause, during which humans are acyclic, FSH is continuously high and estrogen levels are continuously low. This process appears to be fairly unique

to humans and some higher primates, as nonhuman primates such as rhesus monkeys may continue to menstruate normally until the end of their life (Gore *et al.*, 2004; Woller *et al.*, 2002). On the other hand, rodents experience changes in their ovarian hormonal profile similar to those of humans. However unlike in human menopause, this decline in estrogen occurs in rats without corresponding changes in the ovary or ovarian follicular stores. Endocrine aging in the rat occurs over time in a multistep fashion. During middle age they develop irregular cycles characterized by a prolonged estrus phase which eventually leads to acyclicity and decreased fertility. An initial acyclic stage of persistent estrus (chronically elevated mid-cycle estrogen levels, cornified vaginal cells) is followed by persistent diestrus (chronically low levels of estrogen, leukocytic vaginal cytology). Thus, rats are considered to be a good model of hormonal aging as they exhibit similar characteristics to reproductive aging in women including gradual cessation of spontaneous reproductive cycles and decreased fertility (Huang *et al.*, 1975; LeFevre *et al.*, 1988).

In addition to studies examining the natural progression of menopause, the effects of estrogen replacement with surgical menopause are of importance to human medicine. Surgical menopause via bilateral ovariectomy, followed by treatment with estrogen or placebo implants, aids in further examining the role of estrogen in differing hormonal ages. Another model which is commonly used examine the effects of estrogen on pre- and postmenopausal women utilizes rats of differing age groups approximating key stages of hormonal aging women (Bake *et al.*, 2004; Johnson *et al.*, 2005; Sohrabji,

2005). Mature multigravid (MA) rats aged 4-6 months who are proven breeders with previous successful pregnancies and normal cycles are similar to pre-menopausal women. Post-menopausal women are modeled by reproductively senescent (RS) rats aged 9-15 months who are retired breeders with previous successful pregnancies and display acyclicity vaginal cytology confirming constant diestrus. This has been commonly used as model for menopause in women (Bake *et al.*, 2004; Johnson *et al.*, 2005; Sohrabji, 2005).

2.2.2.6. Sexual dimorphism in health and disease

Many human diseases have sex-specific differences in prevalence, age of onset and/or severity (Ober *et al.*, 2008; Patsopoulos *et al.*, 2007). Well-recognized examples include cardiovascular disease (higher predominance in men until women reach menopause) (Choi *et al.*, 2007), asthma (predominant in boys until girls reach puberty) (Postma, 2007), autoimmune diseases, depression, and Alzheimer disease (higher in women throughout life) (Andersen *et al.*, 1999; Gater *et al.*, 1998; Lockshin, 2006), and schizophrenia, Parkinson disease and colorectal cancer (more common in men) (Aleman *et al.*, 2003; Matanoski *et al.*, 2006; Wooten *et al.*, 2004). The aging process is associated with marked sexual dimorphism in the incidence of human neurological and vascular diseases, but the reasons for these sex differences in disease are unclear (Sullivan *et al.*, 1996).

2.2.2.7. Beneficial/protective effects of estrogen

As described in detail previously, estrogen plays a fundamental role in the maintenance of both neuronal and vascular health in women. In younger women, endogenous estrogens clearly exert beneficial effects on neuronal and vascular function. Epidemiological studies suggest that the risk of stroke is lower in premenopausal women (Kawas *et al.*, 1997; Levy *et al.*, 1988; Messerli *et al.*, 1987). Additionally, experiments using animal models have described the protective effects of estrogen (Farhat *et al.*, 1996; Karas, 2002; Simpkins *et al.*, 1997). An abundance of evidence from experimental animal studies has established that estrogen exerts beneficial or protective effects on the cerebrovasculature by reducing vascular reactivity and thereby increasing blood flow through nitric oxide- and vasodilator prostanoid-dependent mechanisms (Geary *et al.*, 2000a; Geary *et al.*, 1998; McNeill *et al.*, 1999; McNeill *et al.*, 2002; Orshal *et al.*, 2004; Osanai *et al.*, 2000; Ospina *et al.*, 2003; Ospina *et al.*, 2002). Additionally, intact young female rats experience less brain injury following stroke than males, yet this protective effect is lost with the removal of estrogen via ovariectomy (Dubal *et al.*, 1998; Lisabeth *et al.*, 2012; Rusa *et al.*, 1999). Disruption of this endocrine environment, both during menopause and with advancing age, contributes to dramatic increases in the incidence of neurodegenerative and vascular diseases, especially stroke.

2.2.2.8. Deleterious/detrimental effects of estrogen

While endogenous estrogens, or estrogen replacement therapy following surgical menopause, exert beneficial effects in younger females, estrogen replacement therapy

appears to be detrimental in older, postmenopausal females (Murphy *et al.*, 2003; Rossouw *et al.*, 2002; Wise *et al.*, 2009). Indeed, epidemiological and experimental studies reveal that both age and estrogen replacement therapy increase the risk for stroke and the extent of brain injury following ischemic stroke in aged females (Bath *et al.*, 2005; Sare *et al.*, 2008). Data from randomized large-scale clinical trials by the Women's Health Initiative (WHI) indicate that oral estrogen replacement therapy (conjugated equine estrogen (CEE) plus medroxyprogesterone acetate (MPA)- 16,608 women or CEE alone- 10,739 women) increases ischemic stroke risk by 44% and 55% respectively, in otherwise healthy post-menopausal women (Hendrix *et al.*, 2006; Wassertheil-Smoller *et al.*, 2003). Smaller-scale clinical trials of women with known vascular disease have also shown increased stroke severity with estrogen treatment. In the Women's Estrogen for Stroke Trial (WEST), women in the estrogen treatment group (17β-estradiol 1mg tablet/day- 664 women) showed increased risk of fatal stroke and worse neurologic outcome from stroke after the first cerebral ischemic event (Viscoli *et al.*, 2001). The Heart and Estrogen/progestin Replacement Study (HERS) reported that estrogen treatment (CEE/MPA- 2763 women) did not significantly increase stroke risk nor did it reduce the incidence of coronary or cerebrovascular events in women with coronary heart disease (Hulley *et al.*, 1998). Interestingly, HERS and the subsequent follow-up study HERSII, reported significant increases in the risk of thromboembolic events including deep vein thrombosis and pulmonary embolism, in estrogen treated women as compared to those taking the placebo (Grady *et al.*, 2000; Hulley *et al.*, 2002).

2.2.3. Aims of Project 2

Since the late 1900s, estrogen has been known to exert neuroprotective effects in animal models of cerebral ischemia or stroke. Younger premenopausal women are protected from ischemic stroke as compared to males; yet this protective effect is lost after menopause. Additionally, premenopausal women experience less damage and greater functional and cognitive recovery from neurologic insult than males. Because of these findings, as well as supporting evidence from animal studies, exposure to estrogen was postulated to be neuroprotective. In 2000, an estimated 10 million women were receiving HRT for the alleviation of menopausal symptoms. However, after just a few years, reports from WHI indicated that estrogen therapy significantly increased incidence and severity of stroke. Thus, it is important to examine why estrogen is beneficial and neuroprotective in young animals but not in postmenopausal women. Additionally, further elucidation of the mechanisms' underlying actions of estrogen on cerebrovascular function with advancing age and during health and disease are of great importance.

It is difficult to reconcile the apparent conflict in beneficial vs. deleterious effects of estrogen on neurological and vascular function unless the effects of age are considered. Although the mechanisms underlying the beneficial effects of estrogen on cerebrovascular function have been studied extensively, the mechanisms responsible for age-dependent deleterious effects of estrogen are largely unknown. This lack of understanding emphasizes the importance of examining cellular and molecular

mechanisms underlying the role of age on the deleterious effects of estrogen replacement therapy and endogenous estrogen in the cerebral vasculature. **Thus, the central hypothesis of the proposed research is that:**

Age enhances the deleterious effects of estrogen on the cerebrovasculature and alters the role of constrictor prostanoids in modulating cerebrovascular reactivity.

This hypothesis will be tested by addressing the following specific aims:

Specific Aim 1: Determine the effects of age and estrogen on reactivity to vasoconstrictor substances important in the regulation of cerebrovascular function.

Specific Aim 2: Determine the effects of age and estrogen on basal and agonist-stimulated production of prostanoids PGI_2 and TXA_2.

3. EXERCISE TRAINING ENHANCES MULTIPLE MECHANISMS OF RELAXATION IN CORONARY ARTERIES OF ISCHEMIC HEARTS

3.1. Introduction

The endothelium plays an important role in mediating vascular tone. In response to mechanical stimuli (shear stress, pulsatile pressure) or vasoactive agonists (bradykinin, acetylcholine), endothelium-dependent relaxation occurs through the release of mediators such as nitric oxide and PGI_2, which affect the adjacent vascular smooth muscle in a paracrine manner. In addition, a third endothelium-derived dilator relaxes vascular smooth muscle by causing hyperpolarization of the underlying smooth muscle cells. While a single EDHF has not been elucidated, numerous candidate mechanisms or pathways have been proposed. BK_{Ca} channels are a common downstream effector for several of these potential EDHFs (Archer *et al.*, 2003; Liu *et al.*, 2011), as well as for nitric oxide (Mistry *et al.*, 1998) and PGI_2 (Burnette *et al.*, 2006). BK_{Ca} channels are prominent in coronary vascular smooth muscle cells and thus, small changes in open probability have significant effects on membrane potential and vasomotor tone (Brayden *et al.*, 1992; Ko *et al.*, 2008).

Endothelial dysfunction is characterized by impaired vasodilation, enhanced vasoconstriction, cell proliferation, platelet activation, vascular permeability and inflammation and aggregation of platelets. In humans, all major cardiovascular risk factors, including hypercholesterolemia, hypertension, diabetes, and smoking, have been

associated with endothelial dysfunction and impaired nitric oxide bioavailability (Heitzer *et al.*, 2001). A primary feature of endothelial dysfunction is the inability of arteries and arterioles to dilate fully in response to vasoactive agonists (Feletou *et al.*, 2006). However, other vasodilatory pathways have been shown to be augmented in conditions in which a reduction in nitric oxide availability exists (Bauersachs *et al.*, 1996; Goto *et al.*, 2012; Miura *et al.*, 2001; Park *et al.*, 2008). This redundancy in vasodilator signaling pathways allows for compensation if one mechanism is impaired.

The effect of exercise on the vascular health of patients with coronary artery disease is of considerable interest. Moderate exercise training has been shown to markedly improve myocardial perfusion and cardiac contractile function in compromised myocardium of diseased patients (Hambrecht *et al.*, 2003; Hambrecht *et al.*, 2000). Previous studies have shown that exercise training enhances eNOS mRNA expression in coronary arteries (Sessa *et al.*, 1994) and eNOS protein levels in coronary arterioles (Laughlin *et al.*, 2001) of control animals. Exercise training also has been shown to increase nitric oxide generation, eNOS (Graham *et al.*, 2004; Grijalva *et al.*, 2008; Tanabe *et al.*, 2003) and p-eNOS (Ser1179) gene expression in animal models of disease (Heaps *et al.*, 2006; Zhou *et al.*, 2010) and in human (Ser1177) coronary artery disease patients (Hambrecht *et al.*, 2003).

Despite evidence that exercise training restores nitric oxide-dependent relaxation in the coronary circulation, little is known about adaptations in other endothelium-dependent

signaling pathways that may function to compensate for reduced nitric oxide bioavailability. In the current study, the hypothesis was that exercise training increases the contribution of multiple mediators to endothelium-mediated relaxation of coronary arteries in the underlying setting of chronic coronary artery occlusion. Mechanistic adaptations in response to chronic coronary artery occlusion, as well as subsequent exercise training were investigated.

3.2. Methods

3.2.1. Ethical approval

All animal protocols were in accordance with "U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training" as detailed in the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" and approved by the Institutional Animal Care and Use Committee at Texas A&M University in accordance with Association for the Assessment and Accreditation of Laboratory Animal Care procedures.

3.2.2. Experimental animals and surgical procedures

As described in detail previously (Heaps *et al.*, 2000), adult female Yucatan miniature swine (Sinclair Research Center, Auxvasse, MO) were surgically instrumented with an ameroid constrictor around the proximal LCX coronary artery. Animals were preanesthetized with glycopyrrolate (0.004 $mg \cdot kg^{-1}$ i.m.), midazolam (0.5 $mg \cdot kg^{-1}$ i.m.), and ketamine (20 $mg \cdot kg^{-1}$ i.m.). Surgical anesthesia was induced with 3% isoflurane.

Animals were intubated and anesthesia maintained with 2-3% isoflurane, balance O_2 throughout aseptic surgery. During the surgery, pigs received the following drugs as necessary: pancuronium or vecuronium bromide (0.1 mg·kg^{-1}; neuromuscular blockers) and lidocaine (1 mg·kg^{-1}, i.v.; antiarrythmic). Immediately following surgery, pigs received ketofen (3.0 mg·kg^{-1}, i.v.; NSAID). Prior to surgery and during surgical recovery, animals received either buprenorphine hydrochloride (0.1 mg·kg^{-1}, i.v.; analgesic) or butorphanol tartrate (0.5 mg·kg^{-1}; i.v., analgesic) every 3-6 hr, as needed for pain relief. Antibiotics (Naxcel, 4 mg·kg^{-1}, i.m.) were administered 24 hours before surgery, immediately prior to surgery and for two days following surgery.

3.2.3. Exercise training

Eight weeks postoperatively, pigs were randomly assigned to either sedentary (n=39) or exercise training (n=38) protocols, in which pigs underwent a progressive treadmill exercise training program 5 days/week for 14 weeks or remained confined to their pens. By week 12 of the progressive exercise program, animals were running 85 minutes/day, 5 days/week, which was maintained throughout the remainder of the training regimen, as described in detail previously (Heaps *et al.*, 2000). Efficacy of the exercise training regimen was verified by comparison of skeletal muscle citrate synthase (oxidative enzyme) levels (Srere, 1969) and heart-to-body weight ratio at conclusion of the study.

3.2.4. Preparation of coronary arteries

At the completion of the 14-week exercise training or sedentary protocols, pigs were anesthetized using rompun (2.25 $mg \cdot kg^{-1}$, i.m.), ketamine (35 $mg \cdot kg^{-1}$, i.m.) and pentothal sodium (30 $mg \cdot kg^{-1}$, i.v.), followed by administration of heparin (1000 $U \cdot kg^{-1}$, i.v.). Pigs were intubated and ventilated with room air and a left lateral thoracotomy was performed in the fourth intercostal space. The heart was removed and placed in iced Krebs bicarbonate buffer (0–4°C) and weighed. Under a dissection microscope, the LAD (nonoccluded) and LCX (collateral-dependent) coronary arteries were isolated and cleaned of myocardium and connective tissue. Visual examination of the ameroid occluder during dissection of the LCX artery indicated complete occlusion in all animals that were included in this study. Complete occlusion has also been verified previously by angiography (Zhou *et al.*, 2010).

3.2.5. Isometric tension studies

Nonoccluded LAD and collateral-dependent LCX coronary arteries were cut into size-matched rings (axial length ~3-4 mm). Rings were mounted for isometric tension studies and lowered into a 20-mL vessel chamber containing Krebs buffer (in mM: 131.5 NaCl, 5 KCl, 1.2 NaH2PO$_4$, 1.2 $MgCl_2$, 3.5 $CaCl_2$, 11.2 glucose, 13.5 $NaHCO_3$, and 0.025 EDTA) at 37.5°C aerated with 95% O_2- 5% CO_2. Coronary rings were individually stretched to optimal length (L_{max}) as determined by repeated exposure to 60 mM KCl at increasing lengths. L_{max} was defined as the length at which a ≥ 5% increase in length produced an increase in tension ≤ 10%. Arteries were pretreated with pharmacological

agents indicated below for 20 min and then preconstricted with $PGF_{2\alpha}$ (30 μM) until steady state contraction was achieved. Concentration-response relationships to bradykinin (10^{-11} to 10^{-6} M) or nitroprusside (10^{-9} to 10^{-4} M) were determined by cumulative addition in half-log increments directly into the tissue bath. Inhibitors included: (1) NOS inhibitor (L-NAME, 300 μM), (2) prostanoid inhibitor (INDO, 5 μM), and (3) BK_{Ca} channel blocker (IBTX, 100 nM).

3.2.6. Endothelial cell dissociation

Endothelial cells were enzymatically dissociated from segments of collateral-dependent LCX and nonoccluded LAD coronary arteries (~1.0 mm luminal diameter). Artery segments were cut longitudinally and pinned lumen side up in low-Ca^{2+} (0.1 mM) physiological buffer containing 294 $U \cdot mL^{-1}$ collagenase, 5 $U \cdot mL^{-1}$ elastase, 2 $mg \cdot mL^{-1}$ BSA, 1 $mg \cdot mL^{-1}$ soybean trypsin inhibitor, and 0.4 $mg \cdot mL^{-1}$ DNase I in a 37 °C water bath for 20 min. The enzyme solution was then replaced with enzyme-free low-Ca^{2+} solution, and isolated cells obtained by repeatedly directing a stream of low-Ca^{2+} solution over the artery via fire-polished Pasteur pipette. Enzymatically dissociated cells were collected and transferred to a 15-mL conical tube. The cells were centrifuged (Sorvall RT7 Plus; swinging bucket rotor RTH-750; 800 rpm) for 3 min, supernatant was removed, and the pellet was resuspended in enzyme-free low-Ca^{2+} solution.

3.2.7. Detection of bradykinin-stimulated nitric oxide levels in isolated endothelial cells

Bradykinin-stimulated nitric oxide levels were measured from freshly isolated endothelial cells in real-time using the fluorescent indicator, DAF-FM DA (Molecular Probes). Cells were incubated with DAF-FM DA (2.5 μM) for 10 min in the dark at room temperature, centrifuged (800 rpm) for 3 min, supernatant removed, and the pellet resuspended in enzyme-free low-Ca^{2+} solution. Cells were pipetted into a superfusion chamber and observed using an epifluorescence microscopy system, which permitted evaluation of DAF fluorescence from multiple user-selected endothelial cells simultaneously throughout the experimental protocol (NIS-Elements AR 3.0). Endothelial cells were morphologically distinguishable from other cell types in the dispersion as characterized previously (Wagner-Mann *et al.*, 1992). Cells were excited with a 175-W xenon arc lamp with a 475-nm interference filter for excitation wavelength (Sutter Instruments; Lambda DG-4). Fluorescence emission was captured at 530 nm every 15 sec and reflected to an interline transfer, progressive-scan, cooled charge-coupled device video camera (CoolSNAP HQ; Photometrics) with a dichroic mirror. The microscope was equipped with an x40 oil immersion objective with a numerical aperature of 1.3. After a three minute baseline period, bradykinin (10^{-9}, $10^{-7.5}$, or 10^{-6} M) was superfused at five minute intervals. To determine delta peak fluorescence, baseline fluorescence was subtracted from the peak fluorescence response to each bradykinin concentration for each cell.

3.2.8. Measurement of PGI_2 levels

Nonoccluded and collateral-dependent arteries were cut into rings (~1 mm axial length), and placed in cold Krebs (4 ˚C) on ice for 30 min. Supernatant was removed and fresh Krebs (± INDO or L-NAME) was added and slowly warmed in a 37 °C water bath. Supernatant was once again removed and replaced with 175 µL of Krebs containing the following agonists/antagonists: 1) control, 2) INDO (5 µM), 3) bradykinin (10^{-6} M), 4) INDO (5 µM) + bradykinin (10^{-6} M), or 5) L-NAME (300 µM) + bradykinin (10^{-6} M). Following a 10-min incubation period at 37 °C, rings were removed from solution. Arterial rings and supernatant were snap-frozen in separate tubes. Supernatant samples were prepped in accordance with the protocol provided with the 6-keto Prostaglandin $F_{1\alpha}$ EIA kit (Cayman Chemical, no. 515211) and diluted 1:20 immediately prior to addition to the 96-well plate. BCA protein assay kit (Thermo Scientific Pierce) was used to determine total protein concentration of arterial rings.

3.2.9. Smooth muscle cell dissociation

All electrophysiology experiments were performed using freshly dispersed arterial smooth muscle cells from nonoccluded and collateral-dependent arteries. Coronary arteries were pinned lumen side up in low-Ca^{2+} (0.1 mM) physiological buffer containing 1.4 mg/ml papain, 0.4 mg/ml DTT, 0.4 mg/ml BSA. Cells were enzymatically dissociated by incubation in a 37 °C water bath for 30-45 min. The enzyme solution was then replaced with enzyme-free low-Ca^{2+} solution and the arteries dispersed with gentle trituration by micropipette for isolation of single smooth muscle

cells. Smooth muscle cells were morphologically distinguishable from other cell types as described previously (Wagner-Mann *et al.*, 1992). Isolated cells were maintained in low-Ca^{2+} solution at 4 °C until use (0-6 h).

3.2.10. Whole-cell voltage clamp

K^+ channel currents were determined using standard whole-cell voltage-clamp technique as used routinely by Heaps' laboratory (Heaps *et al.*, 2008; Heaps *et al.*, 2005; Xie *et al.*, 2013). All proposed experiments were performed using freshly dispersed smooth muscle cells on the day of animal termination. Cells were initially superfused with low-Ca^{2+} PSS containing: (in mM) 138 NaCl, 5 KCl, 0.1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 20 HEPES, pH 7.4, during gigaseal formation. Heat-polished glass pipettes (2-5 MΩ) were filled with a solution containing (in mM): 120 KCl, 10 NaCl, 1 $MgCl_2$, 10 EGTA, and 10 HEPES, pH 7.1, with KOH. Ionic currents were amplified by an Axopatch 200B patch-clamp amplifier (Axon Instruments). Cells were continuously perfused under gravity flow at room temperature (22-25 °C). IBTX-sensitive currents were obtained by subtraction of currents in the presence of IBTX from control currents in smooth muscle cells isolated from nonoccluded and collateral-dependent arteries of sedentary and exercise-trained animals.

3.2.11. Immunoblot for BK_{Ca} channel

Arterial rings (~3 mm length; ~1.0 mm diameter) were isolated from both the collateral-dependent LCX and nonoccluded LAD, quick frozen and stored at -80 °C for later

immunoblot analysis, as described in detail previously (Fogarty *et al.*, 2009). Arterial lysate (20 μg total protein) was subjected to SDS–PAGE (4-20 % gradient gel), transferred to PVDF membrane, and probed overnight with primary antibody. Primary antibodies included BK_{Ca} channel alpha subunit (Alomone Labs, APC-021, 1:200) and smooth muscle α-actin (Abcam, ab21027, 1:1,000).

3.2.12. Statistics

Animal body weight, heart-to-body weight, IC_{50}, and citrate synthase activity were compared between sedentary and exercise trained pigs using student's t-test. Dimensional characteristics of coronary arteries, fluorescence (DAF-FM DA), PGI_2, and immunoblot data were compared using two-way ANOVA. Bradykinin-mediated relaxation and whole-cell K^+ currents were evaluated by repeated measures two-way ANOVA and the Greenhouse-Geisser adjustment to control for type I error due to unequal group sizes (Ludbrook, 1994). If a main effect was identified by ANOVA, Bonferroni tests were used to detect individual differences. A P value ≤ 0.05 was considered significant. "n" values reflect the number of animals studied. When more than one coronary arterial ring from the nonoccluded or collateral-dependent region of a given animal was used in identical protocols, the responses from those rings were averaged before data analyses were conducted.

3.3. Results

3.3.1. Efficacy of the exercise-training program

Effectiveness of the 14-week exercise-training regimen was demonstrated by significant increases in skeletal muscle oxidative enzyme activity and an increased heart-to-body weight ratio in exercise-trained compared with sedentary animals. Citrate synthase activity increased significantly ($P \leq 0.02$ for all comparisons) in the deltoid muscle (45.6 ± 1.4 vs. 36.9 ± 1.3 $\mu mol \cdot min^{-1} \cdot g^{-1}$) and lateral (38.7 ± 1.1 vs. 32.9 ± 1.0 $\mu mol \cdot min^{-1} \cdot g^{-1}$), medial (43.0 ± 1.8 vs. 36.3 ± 1.5 $\mu mol \cdot min^{-1} \cdot g^{-1}$), and long (34.7 ± 1.6 vs. 29.7 ± 1.2 $\mu mol \cdot min^{-1} \cdot g^{-1}$) heads of the triceps brachii muscle in exercise-trained (n=38) compared with sedentary (n=39) pigs. Although body weight did not differ between sedentary and exercise-trained animals at the time of death (34.42 ± 0.77 kg vs. 33.80 ± 0.75 kg), heart-to-body weight ratio was significantly greater in exercise-trained compared with sedentary pigs (4.47 ± 0.10 vs. 5.27 ± 0.10; $P \leq 0.001$).

3.3.2. Coronary artery dimensions and characteristics

Analyses of dimensional characteristics of arterial rings used for isometric tension studies revealed that the mean luminal diameter of collateral-dependent LCX rings was statistically smaller than nonoccluded LAD rings of sedentary pigs (Table 1). Additional artery dimensions, including outer diameter, wall thickness and axial length were not significantly different between groups. Furthermore, the resting tension of coronary ring segments at L_{max} was not significantly different between the four artery treatment groups.

Table 1. Dimensional characteristics of coronary arteries.

Group	n	Outer Diameter (mm)	Lumen Diameter (mm)	Wall Thickness (mm)	Axial Length (mm)	RT L_{max}
Sedentary						
nonoccluded	59	1.52 ± 0.05	1.07 ± 0.04	0.21 ± 0.01	3.29 ± 0.03	1.34 ± 0.10
collateral-dependent	55	1.37 ± 0.05	0.92 ± 0.04[a]	0.21 ± 0.01	3.23 ± 0.04	1.28 ± 0.11
Exercise-trained						
nonoccluded	54	1.49 ± 0.04	1.04 ± 0.03	0.21 ± 0.01	3.25 ± 0.03	1.23 ± 0.08
collateral-dependent	54	1.37 ± 0.05	0.94 ± 0.04	0.20 ± 0.01	3.26 ± 0.04	1.29 ± 0.11

Values are means ± SEM. RT L_{max}, resting tension at optimal length where maximal active tension to KCl-induced depolarization is developed. n= number of animals studied. [a]$P \leq 0.05$ vs. sedentary nonoccluded

3.3.3. Contribution of nitric oxide, PGI_2 and BK_{Ca} channel to resting tension

Incubation of coronary arterial rings with the NOS inhibitor, L-NAME, resulted in increases in resting tension that were not statistically different between treatment groups (Figure 6A). Combined NOS and prostanoid inhibition (L-NAME+INDO) significantly increased resting tension of collateral-dependent arteries of exercise-trained pigs compared with all other treatment groups (Figure 6B). Importantly, comparison of panels A and B reveals that L-NAME+ INDO attenuated the increase in resting tension generated by L-NAME alone in all treatment groups except collateral-dependent arteries of exercise-trained pigs. These data suggest that constricting rather than dilating prostanoids contribute to resting tone in the three other treatment groups, while prostanoids do not appear to contribute to basal tension in collateral-dependent arteries

of exercise-trained pigs. Addition of IBTX had negligible effect on resting tension suggesting little role of BK_{Ca} channels on basal tone.

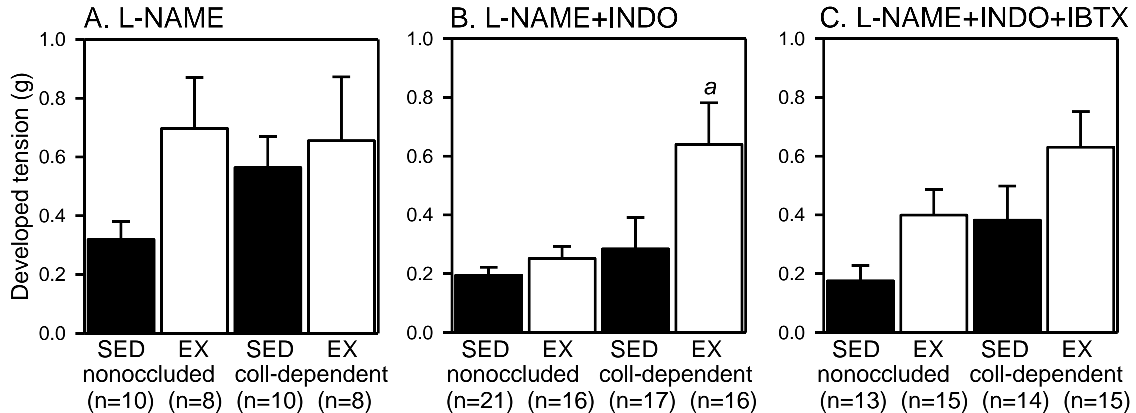

Figure 6. Effects of chronic occlusion and exercise training on the contribution of nitric oxide, prostanoids, and BKCa channel to resting tension.
Increase in baseline tension in nonoccluded and collateral-dependent arteries of sedentary (SED) and exercise-trained (EX) pigs to L-NAME (300 μM) (A), L-NAME+INDO (5 μM) (B), and L-NAME+INDO+IBTX (100 nM) (C). Values are means ± S.E.M. of the number of animals in parentheses. [a] vs. all other treatments within panel; $p \leq 0.05$.

3.3.4. Effects of exercise training and chronic occlusion on bradykinin-mediated relaxation

The effects of exercise training and chronic coronary occlusion on bradykinin-mediated relaxation are shown in Figure 7 and Table 2. Comparison of control curves across panels revealed no significant difference in relaxation from nonoccluded and collateral-dependent coronary arteries of either sedentary or exercise-trained animals. Concurrent studies were performed to determine the contribution of nitric oxide, prostanoids, and

BK_{Ca} channels to bradykinin-mediated relaxation. NOS inhibition significantly attenuated relaxation in all groups compared with the control curve, however, relaxation responses remained significantly more persistent in arteries from exercise-trained compared with sedentary pigs. Similarly, sensitivity (IC_{50}, Table 2) of the arterial rings to bradykinin was significantly reduced following pretreatment with L-NAME, although the reduction in sensitivity was less pronounced in the arteries of exercise-trained pigs. Combined inhibition of NOS and prostanoids partially reversed the enhanced relaxation observed in exercise-trained pigs after NOS inhibition alone, however, significantly increased relaxation continued to persist in both nonoccluded and collateral-dependent arteries of exercise-trained animals. Sensitivity (Table 2) of the arterial rings to bradykinin was not further reduced following pretreatment with L-NAME + INDO compared with L-NAME alone in all groups. Finally, combined blockade of NOS, prostanoids and BK_{Ca} channels ablated the enhanced exercise training-mediated relaxation in both nonoccluded and collateral-dependent arteries such that relaxation responses were similar across treatment groups when all three mediators were inhibited. Sensitivity (Table 2) of the arterial rings to bradykinin was not further reduced following pretreatment with L-NAME + INDO + IBTX compared with L-NAME alone or L-NAME + INDO.

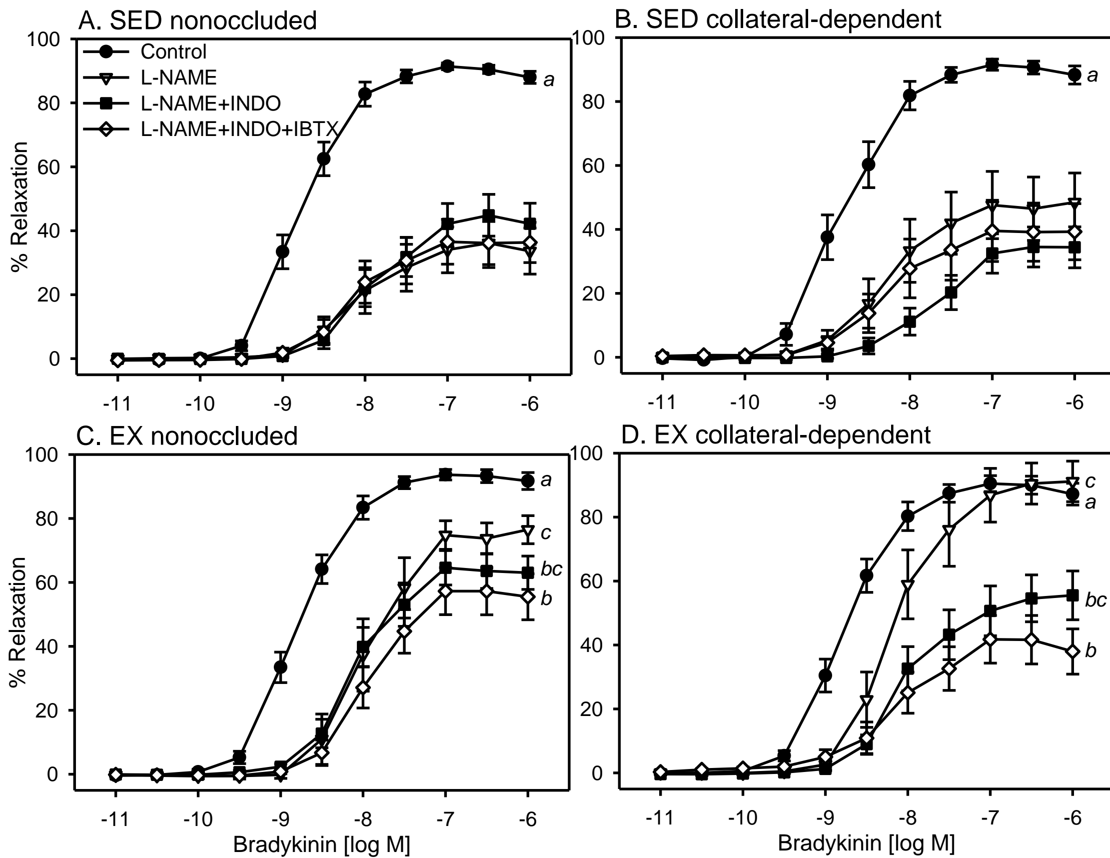

Figure 7. Effects of chronic occlusion and exercise training on bradykinin mediated dilation. Relaxation-responses of arteries from nonoccluded control or collateral-dependent, arteries of sedentary (A, B) or exercise trained (C, D) pigs in response to increasing concentrations of bradykinin in the absence and presence of inhibitors. Values are means ± S.E.M. of the number of animals in parentheses. [a] vs. all other treatment groups within panel; [b] vs. L-NAME curve within panel; [c] vs. SED curve across panels; $p \leq 0.05$.

Table 2. IC_{50} values (log M) for bradykinin-mediated relaxation in the absence and presence of inhibition of select signaling pathways.

Group	Control	L-NAME	L-NAME + INDO	L-NAME + INDO + IBTX
Sedentary				
nonoccluded	-8.73 ± 0.09	-7.92 ± 0.14[a]	-7.72 ± 0.10[a]	-7.83 ± 0.16[a]
collateral-dependent	-8.75 ± 0.11	-7.67 ± 0.30[a]	-7.62 ± 0.11[a]	-8.10 ± 0.27
Exercise-trained				
nonoccluded	-8.73 ± 0.06	-7.87 ± 0.16[a]	-7.96 ± 0.11[a]	-7.95 ± 0.17[a]
collateral-dependent	-8.73 ± 0.08	-8.07 ± 0.18[a]	-7.94 ± 0.13[a]	-7.97 ± 0.10[a]

Values are means ± SEM; n values are same as indicated in respective concentration-response curves from Figure 7. [a]$P \leq 0.05$ vs. corresponding IC_{50} from control (no inhibitors).

3.3.5. Smooth muscle responsiveness to nitroprusside

The response of coronary arterial rings to the endothelium-independent nitric oxide donor, nitroprusside was also evaluated in this study. Concentration-dependent relaxation responses to nitroprusside were not significantly different in arteries from the collateral-dependent or nonoccluded arteries of sedentary or exercise-trained pigs (IC_{50} -6.81 ± 0.08 vs. -6.58 ± 0.11 vs. -6.57 ± 0.09 vs. -6.46 ± 0.15 log M, respectively). These data suggest that smooth muscle responsiveness to nitric oxide was not altered by exercise training or chronic occlusion.

3.3.6. Measurement of bradykinin-stimulated nitric oxide

Bradykinin-mediated changes in intracellular nitric oxide levels were evaluated in isolated endothelial cells from both the collateral-dependent and nonoccluded arteries (Figure 8). There were no differences in peak delta nitric oxide levels in response to low

concentrations of bradykinin (10^{-9} M) across treatment groups. In contrast, higher concentrations of bradykinin ($10^{-7.5}$ and 10^{-6} M) stimulated significantly enhanced nitric oxide levels in cells isolated from both control and collateral-dependent arteries of exercise-trained compared with sedentary pigs.

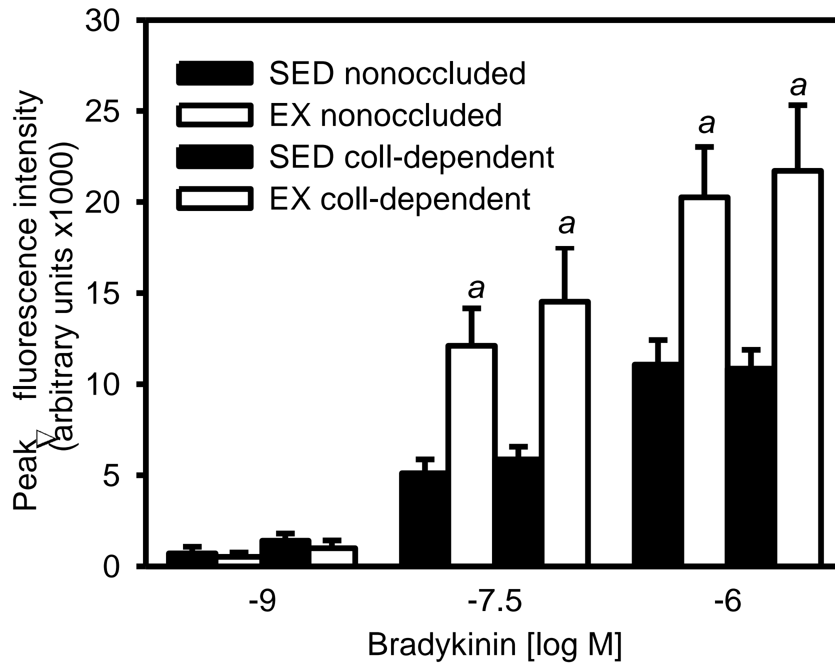

Figure 8. Effects of chronic occlusion and exercise training on nitric oxide levels. Exercise training significantly enhanced nitric oxide levels in response to bradykinin ($10^{-7.5}$ and 10^{-6} M) in endothelial cells isolated from both nonoccluded LAD and collateral-dependent LCX arteries compared with cells from sedentary pigs. Values are means ± S.E.M. number of animals, cells in parentheses. [a] $p \leq 0.05$ vs. respective SED value; $p \leq 0.05$.

3.3.7. Measurement of PGI_2

Both basal and bradykinin-stimulated changes in intracellular PGI_2 levels in arterial rings of both the collateral-dependent LCX and nonoccluded LAD were evaluated (Figure 9). PGI_2 levels were assessed by measurement of the stable end-product of PGI_2 metabolism, 6-keto-prostaglandin $F_{1\alpha}$. Basal PGI_2 levels were not significantly altered by chronic occlusion or exercise training. There was no difference in PGI_2 release with bradykinin (10^{-9} M) stimulation (data not shown) however, bradykinin (10^{-6} M) caused a significant increase in PGI_2 levels in all arterial treatment groups. In order to examine the specificity of the assay, a subset of arterial rings were pretreated with the COX inhibitor, INDO (5 μM). Pretreatment with INDO confirmed the specificity of the assay and caused a significant decrease in both basal and bradykinin-stimulated PGI_2 levels (Figure 5). To assess the possibility of cross-talk between nitric oxide and PGI_2, PGI_2 levels in the presence of NOS inhibition were examined. Interestingly, pretreatment with L-NAME showed a slight tendency to enhance bradykinin-stimulated PGI_2 in the exercise-trained group, suggesting nitric oxide may hinder the exercise-trained enhancement of PGI_2 production.

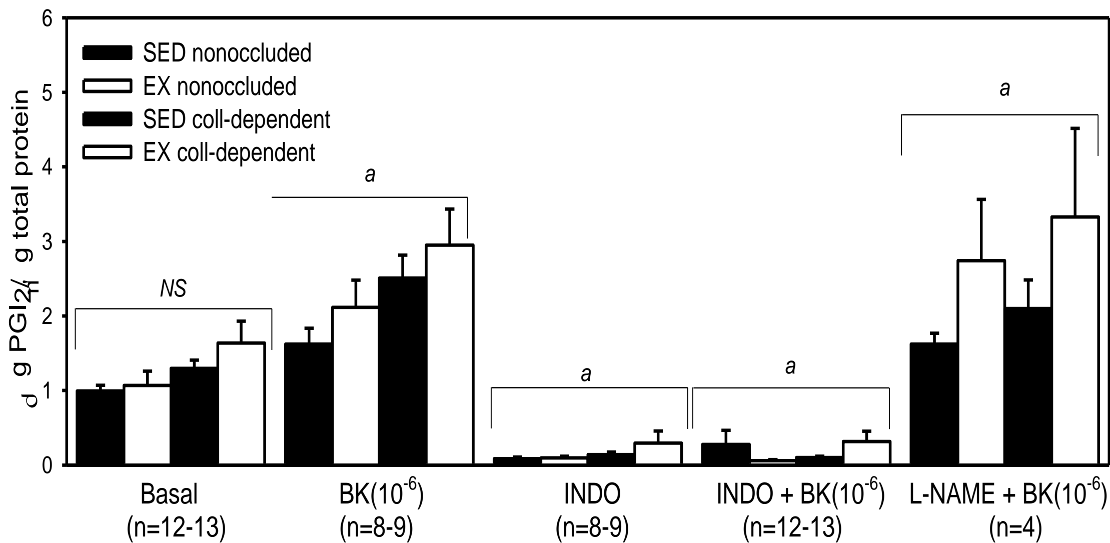

Figure 9. Effects of chronic occlusion and exercise training on PGI_2 levels.
Basal PGI_2 was not significantly different between groups. Bradykinin caused a significant increase in PGI_2 in all arterial groups. Treatment with indomethacin (INDO) confirmed the selectivity of the assay and caused a significant decrease in PGI_2 both with and without bradykinin stimulation. Pretreatment with L-NAME tended to further increase bradykinin-stimulated PGI_2 in nonoccluded and collateral-dependent artery rings of exercise trained (EX) compared with sedentary (SED) animals. Values are means ± S.E.M. of the number of animals in parentheses. [a] vs. respective basal value; $p \leq 0.05$.

3.3.8. Whole-cell K^+ channel current

The effects of chronic occlusion and exercise training on coronary smooth muscle K^+ channel currents are shown in Figure 10. Whole-cell currents were elicited by 500-ms step depolarizations to test potentials ranging from -70 to +100 mV from a holding potential of -80 mV. Representative traces for currents of cells from nonoccluded and collateral-dependent arteries of sedentary and exercise-trained pigs are shown in Figure 10A. For current-voltage relationships in Figure 10B and C, the mean value of outward current for last 100 ms of each test potential normalized to cell membrane capacitance

(pA/pF) was plotted. Cell capacitance was not significantly different between smooth muscle cells from nonoccluded and collateral-dependent arteries of sedentary (11.1 ± 0.8 and 11.7 ± 0.9 pF, respectively) and exercise trained (12.0 ± 0.8 and 12.2 ± 0.8 pF, respectively) pigs. Comparison of the current-voltage relationships indicated that neither chronic coronary occlusion nor exercise training altered whole cell K^+ currents in arterial smooth muscle cells.

3.3.9. Iberiotoxin-sensitive K^+ channel current

The effects of chronic occlusion and exercise training on IBTX-sensitive (BK_{Ca}) channel currents are shown in Figure 11. IBTX-sensitive currents were obtained by subtraction of currents in the presence of IBTX from control currents (difference currents) (Figure 11A). Findings from these studies revealed that BK_{Ca} channel currents were not significantly altered by occlusion or exercise training in coronary arterial smooth muscle (Figure 11B and C).

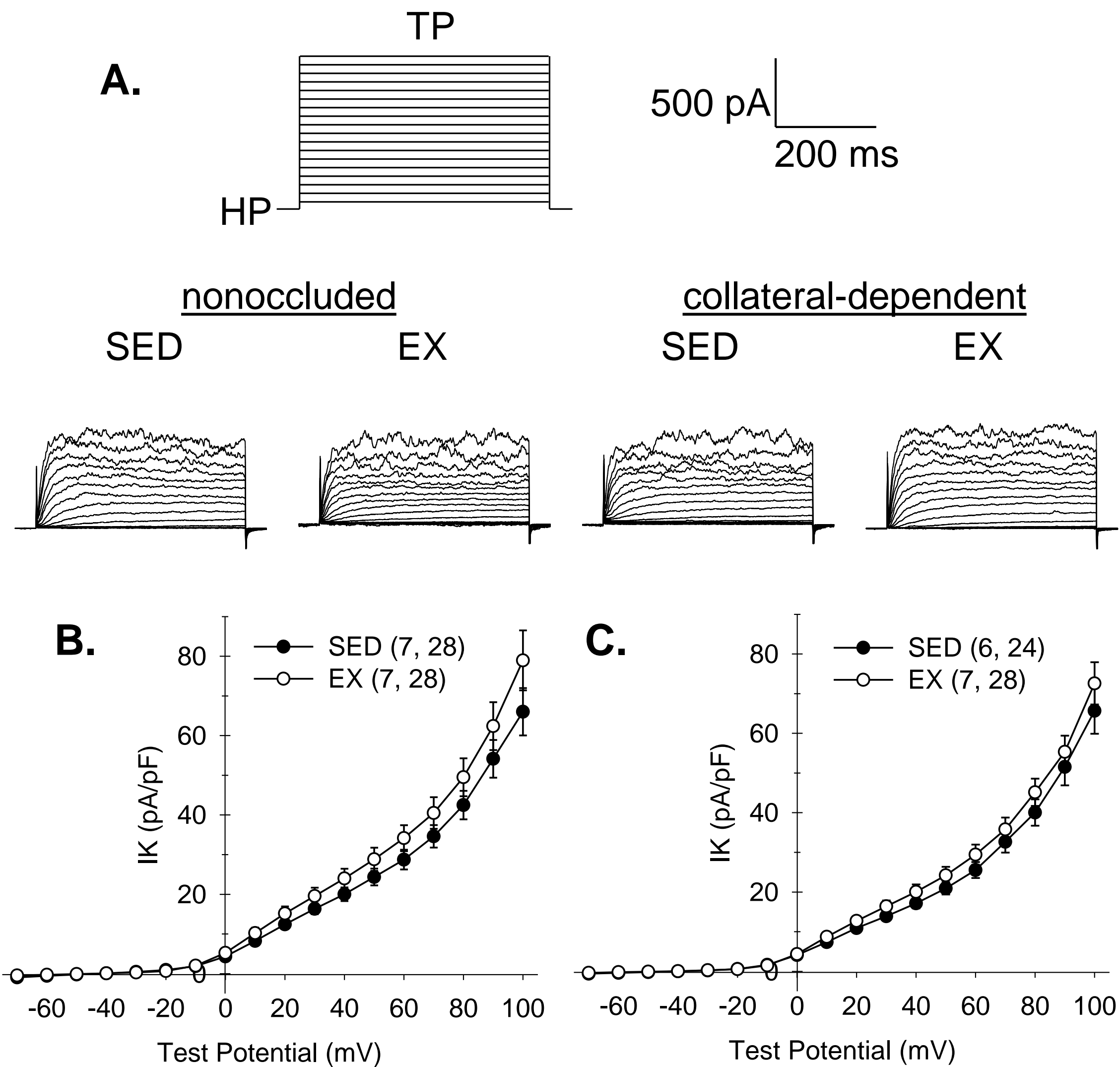

Figure 10. Effects of chronic occlusion and exercise training on whole-cell K+ channel current in coronary artery smooth muscle cells.

Currents were elicited by 500-ms step depolarizations to potentials ranging from -70 to +100 in 10mV increments from a holding potential of -80mV. Representative traces for currents from cells from nonoccluded and collateral-dependent arteries of sedentary and exercise-trained pigs are shown (A). Comparison of current voltage relationships obtained by plotting the mean outward current at the end of each test potential normalized to cell membrane capacitance (B, C). Values are means ± S.E.M. of the number of animals, cells in parentheses; no significant differences existed.

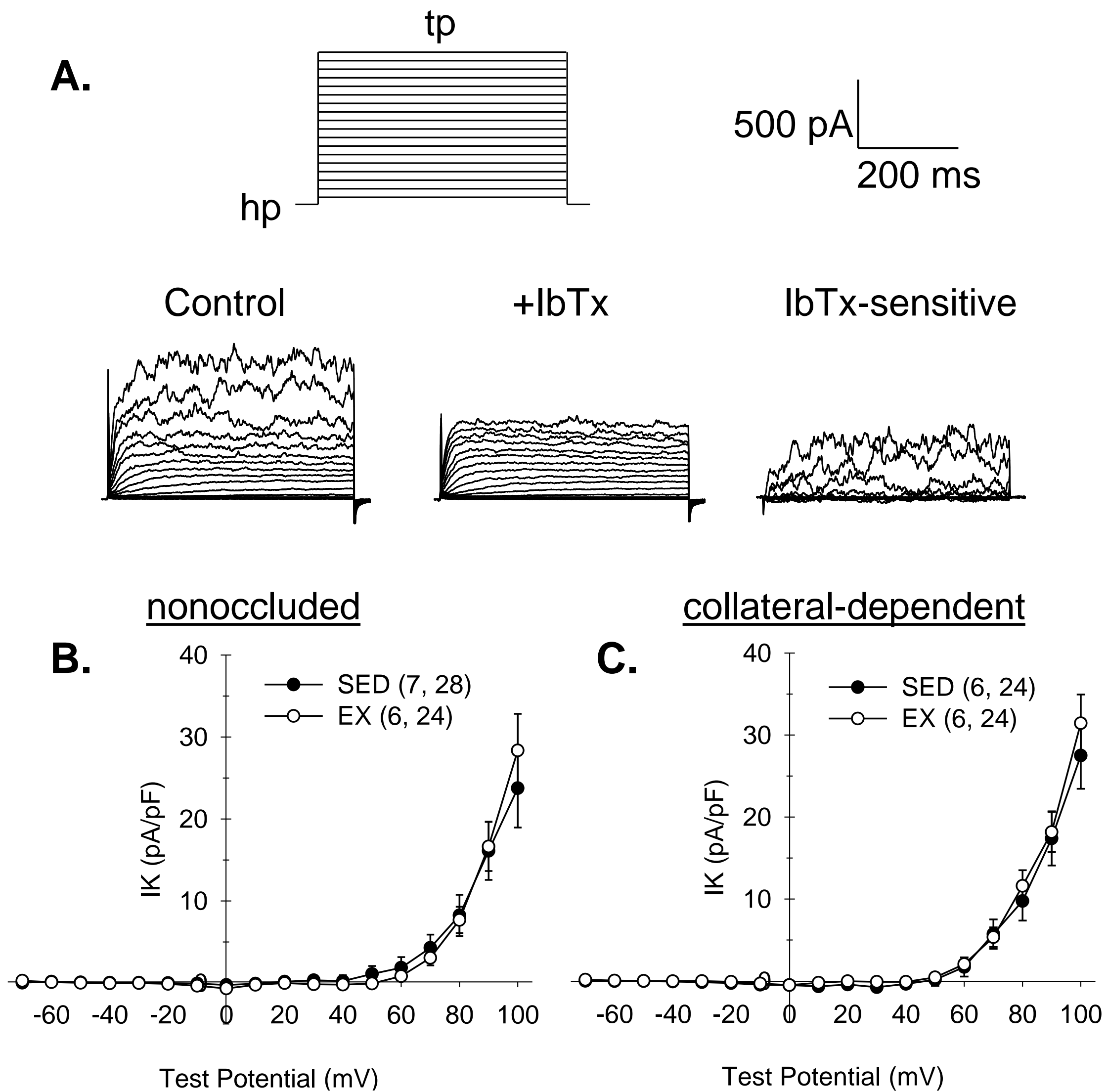

Figure 11. Effects of chronic occlusion and exercise training on IBTX-sensitive K+ channel currents in coronary artery smooth muscle cells.
Currents were obtained as described in Figure 5 legend. IBTX-sensitive K^+ channel currents were obtained by subtraction of currents in the presence of IBTX (100 nM) from control currents (A). Comparison of IBTX-sensitive current -voltage relationships obtained by plotting the mean outward current at the end of each test potential normalized to cell membrane capacitance (B, C). Values are means ± S.E.M. of the number of animal, cells in parentheses; no significant differences existed.

3.3.10. BK_{Ca} channel protein

Determination of protein by immunoblot revealed that neither occlusion nor exercise training significantly altered BK_{Ca} channel protein levels in coronary arteries as shown in Figure 12. The control protein, smooth muscle α-actin, also was not significantly altered by occlusion or exercise training in these arteries.

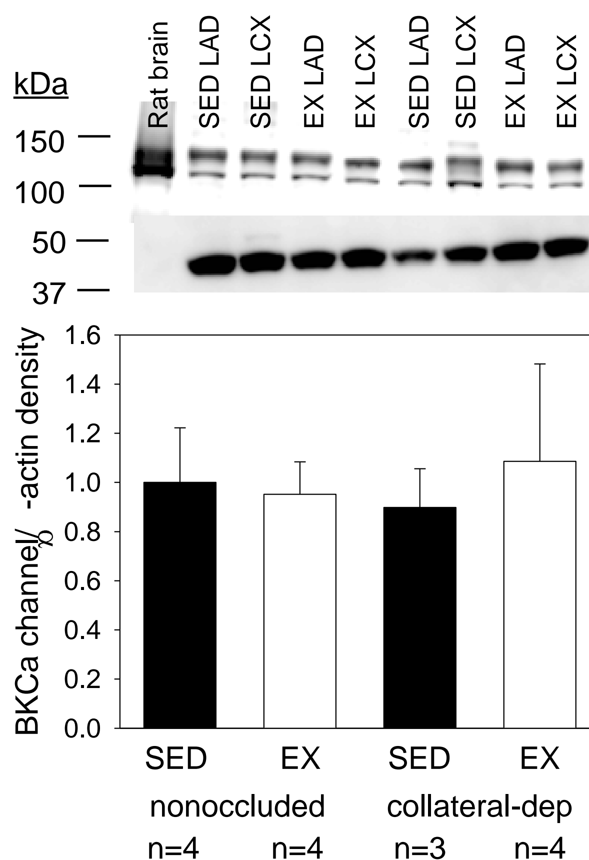

Figure 12. Effects of chronic occlusion and exercise training on BK_{Ca} channel protein levels in coronary arteries.
Immunoblot analyses demonstrated that BKCa channel protein levels were not significantly altered by occlusion or exercise training. Protein was quantified by densitometry analysis, normalized to smooth muscle α-actin, and expressed relative to the density of nonoccluded arteries of SED pigs. Values are means ± SEM of the number of animals indicated. No significant difference existed.

3.4. Discussion

This study reveals the novel finding that exercise training enhances multiple mechanisms of endothelium-dependent vascular relaxation which appear to function in a compensatory manner rather than in an additive fashion. Bradykinin-stimulated nitric oxide levels were significantly increased after exercise training in endothelial cells of both nonoccluded and collateral-dependent arteries. Similarly, PGI_2 levels tended to be increased in arteries of exercise-trained compared with respective arteries of sedentary pigs. Interestingly, despite increases in these signaling molecules with exercise training, bradykinin-mediated, endothelium-dependent relaxation was not enhanced in arteries from exercise-trained pigs. On the other hand, bradykinin-mediated relaxation was more persistent in arteries from exercise-trained pigs after inhibition of select endothelium-dependent signaling molecules, suggesting redundancy in signaling pathways of vascular relaxation.

While NOS inhibition significantly reduced bradykinin-mediated relaxation in all artery treatment groups, the effect was much more marked in arteries of sedentary compared with exercise-trained animals. This finding might be interpreted to indicate that the contribution of NOS to endothelium-mediated relaxation is significantly greater in sedentary compared with exercise-trained pigs or that other pathways of relaxation better compensate for the loss of nitric oxide in the exercise-trained animals. While combined inhibition of nitric oxide and prostanoids partially reversed the difference in bradykinin-mediated relaxation in the presence of NOS inhibition alone, the significantly enhanced

relaxation continued to persist in arteries from exercise-trained pigs. Finally, combined inhibition of nitric oxide, prostanoids, and BK_{Ca} channels eliminated the persistent relaxation observed in arteries from exercise- trained pigs. Taken together, these data suggest that the contributions of nitric oxide, dilating prostanoids, and BK_{Ca} channel all increase after exercise training but that some limitation exists regarding the contribution of these molecules to the overall relaxation response, such as negative modulation between the mediators or their pathways. Indeed, numerous reports have revealed interaction of various signaling pathways that can negatively or positively regulate one another (Bauersachs *et al.*, 1996; Nishikawa *et al.*, 2000; Osanai *et al.*, 2000; Puybasset *et al.*, 1996). Previous studies have reported crosstalk between nitric oxide and PGI_2 in the coronary circulation. In canine coronary arteries, prostanoid inhibition had no effect on bradykinin-mediated relaxation until combined with NOS inhibition (Puybasset *et al.*, 1996) and bradykinin-stimulated PGI_2 release was significantly enhanced by chronic NOS blockade (Beverelli *et al.*, 1997). It is noteworthy that in arteries of exercise-trained pigs, PGI_2 levels in the presence of NOS inhibition were slightly increased above that observed in the absence of NOS inhibition, suggesting that nitric oxide may negatively modulate PGI_2 production.

Another critical aspect of this study was quantifying nitric oxide and prostacyclin levels in response to bradykinin stimulation. These findings demonstrate that bradykinin-stimulated nitric oxide levels were significantly increased in endothelial cells from exercise-trained compared with sedentary pigs. Taken together with the functional data

in the presence of NOS inhibition, these data lend support to the interpretation that other pathways of relaxation better compensate after inhibition of nitric oxide in the exercise-trained animals rather than a lesser contribution of nitric oxide production to relaxation in exercise-trained pigs. These results agree with previous studies from Heaps' laboratory which have reported significant exercise training-induced adaptations in mechanisms that control nitric oxide production in both control and collateral-dependent coronary arteries, resulting in enhanced nitric oxide production (Zhou *et al.*, 2010). Other studies have shown increased eNOS mRNA and protein levels after exercise training in control animals (Laughlin *et al.*, 2001; Sessa *et al.*, 1994) and in numerous animal models of disease (Graham *et al.*, 2004; Grijalva *et al.*, 2008; Heaps *et al.*, 2006; Tanabe *et al.*, 2003; Zhang *et al.*, 2007). Additionally, patients with stable coronary artery disease that underwent an exercise training program, also exhibited improvements in endothelium-dependent dilation and mean peak blood flow velocity concomitant with increased phosphorylation of eNOS Ser1177 and total eNOS protein and mRNA expression in the left internal mammary artery of these patients (Hambrecht *et al.*, 2003).

Although statistical significance was not attained, PGI_2 levels tended to be enhanced in both nonoccluded and collateral-dependent arteries of exercise-trained pigs compared to their respective arteries in sedentary animals. These findings coincide with the functional data which showed a significantly greater effect of combined NOS plus prostanoid inhibition compared with NOS inhibition alone in the exercise-trained but not sedentary pigs. Additionally, exercise-training tended to increase bradykinin-stimulated PGI_2

levels after nitric oxide inhibition in both nonoccluded and collateral-dependent arteries. Taken together, this data suggest that exercise training enhances both nitric oxide and PGI_2 contributions to vasorelaxation and that nitric oxide may have inhibitory actions on PGI_2 production.

While the effects of exercise training on nitric oxide production have been widely studied, exercise training-induced adaptations of PGI_2-dependent responses are not well understood. There is debate on the importance of prostanoids in the regulation of coronary blood flow in humans. Studies of patients with coronary artery disease have shown that indomethacin diminishes vasodilation and reduces resting coronary blood flow (Duffy *et al.*, 1999), suggesting that prostacyclin contributes to blood flow in disease states under resting conditions. In contrast, in healthy patients, prostanoids were not essential to basal coronary flow or to modulation of coronary flow with exercise (Edlund *et al.*, 1989). Taken together, these findings suggest that blunted NOS activity often associated with human disease may be compensated by an increased contribution of prostanoids. In agreement, this study's data clearly suggest that following NOS inhibition, PGI_2 significantly contributes to bradykinin-mediated relaxation in coronary arteries of exercise-trained pigs in the underlying setting of chronic coronary artery occlusion. However, the role of PGI_2 in the absence of NOS inhibition was not examined in these studies. In a canine model of permanent occlusion of the left anterior descending artery, resting myocardial blood flow in both the normal and collateral-dependent zones were not altered by cyclooxygenase blockade (Altman *et al.*, 1995). However, during

exercise, indomethacin produced an increase in transcollateral resistance with was associated with a decrease in subendocardial flow in the collateral-dependent zone (Altman *et al.*, 1995), suggesting a role of PGI_2 to maintain blood flow to compromised regions during stress. Additionally, a single bout of exercise increases PGI_2 metabolites in blood (Frandsen *et al.*, 2000; Zoladz *et al.*, 2009), and interstitial fluid of muscles (Frandsen *et al.*, 2000; Karamouzis *et al.*, 2001). Longer endurance exercise training programs (5-8 weeks) have also found increases in plasma concentrations (Zoladz *et al.*, 2010) of PGI_2 in humans, however the importance of these systemic changes on local vascular function have not yet been determined.

In this study there was no effect of chronic occlusion or exercise-training on K^+ channel currents. These findings agree with previous studies which showed no effect of exercise training on whole-cell K^+ currents of smooth muscle cells from coronary arteries of both control and hypercholesterolemic pigs (Bowles *et al.*, 1998; Heaps *et al.*, 2008). Furthermore, there was no effect of occlusion or exercise-training on BK_{Ca} channel currents in these studies. In contrast, the effect of IBTX in functional data suggests that BK_{Ca} channels do contribute to the enhanced relaxation after exercise. Taken together, these data suggest that a bradykinin-sensitive cellular signaling pathway that mediates smooth muscle relaxation via BK_{Ca} channels may be upregulated by exercise training and contribute to enhanced relaxation. This pathway may be a putative EDHF since the contribution of BK_{Ca} channels persisted in the presence of NOS and prostanoid inhibition. Dr. Heaps' laboratory recently reported that exercise training significantly

enhances the role of the superoxide/H_2O_2 signaling pathway (an EDHF candidate) to endothelium-mediated dilation in collateral-dependent coronary arterioles (Xie *et al.*, 2012). EDHF and PGI_2 have been suggested to act as backup vasodilators, hence their role in dilation may be more relevant after the nitric oxide pathway is compromised (Bauersachs *et al.*, 1996; Nishikawa *et al.*, 2000; Osanai *et al.*, 2000). Indeed, the role of compensatory mechanisms has been shown to be very important in numerous vascular diseases associated with decreases in nitric oxide production. In hypertension, compensation occurs via increased K^+ channel activity and increased release of hyperpolarizing factors (Asano *et al.*, 1993) while in hypercholesterolemia compensatory increases in calcium-dependent potassium channels is evident (Najibi *et al.*, 1994). EDHF has also been shown to compensate for diminished nitric oxide-dependent dilation in numerous disease states including type 2 diabetes (Park *et al.*, 2008), hypertension (Goto *et al.*, 2012), and hyperparathyroidism (Virdis *et al.*, 2010).

3.4.1. Clinical significance and conclusions

Exercise training improves vascular function and contributes to enhanced myocardial perfusion and cardiac contractile function in numerous vascular disease states (Hambrecht *et al.*, 2003; Hambrecht *et al.*, 2000). Despite remarkable evidence for the therapeutic benefits of physical activity, the mechanisms by which regular exercise improves vascular function in the setting of coronary artery disease are not fully understood. The current study provides new evidence that exercise training enhances the contribution of numerous vasodilators, including nitric oxide, PGI_2 and BK_{Ca} channels,

in the underlying setting of coronary artery disease. These exciting findings advance the current understanding of coronary vascular adaptations to exercise in health and coronary artery disease.

4. EFFECTS OF AGE AND SEX ON CEREBROVASCULAR FUNCTION IN THE RAT MIDDLE CEREBRAL ARTERY

4.1. Introduction

The human aging process is associated with marked sexual dimorphism in the incidences of neurological and vascular diseases, but the reasons for these sex differences in disease are unclear (Sullivan *et al.*, 1996). Premenopausal women exhibit lower incidences of cardiovascular disease and stroke than males of the same age, yet after menopause these differences dissipate. Because the risks of cardiovascular disease and stroke increase with the onset of menopause, estrogen has been implicated as protective against these diseases. Indeed, estrogen appears to play a fundamental role in the maintenance of both neuronal and vascular health in younger women and to be protective against diseases such as coronary artery disease, hypertension, and stroke (Geary *et al.*, 2000a; Geary *et al.*, 1998; McNeill *et al.*, 1999; Orshal *et al.*, 2004; Ospina *et al.*, 2003). In numerous animal studies, estrogen exerts beneficial effects by: 1) enhancing vasodilator factors including nitric oxide and prostacyclin; 2) increasing levels of endothelial nitric oxide synthase (eNOS), cycloxygenase-1 (COX-1) and prostacyclin synthase (PGIS) protein expression; and 3) stimulating the phosphatidylinositol 3-kinase/Akt pathway which increases eNOS phosphorylation, eNOS activity, and subsequently nitric oxide production (Duckles *et al.*, 2007).

Disruption of the endocrine environment, both during menopause and with advancing age, contributes to dramatic increases in the incidence of neurodegenerative and vascular diseases, especially stroke. While both endogenous estrogens and estrogen replacement therapy following surgical menopause exert beneficial effects in younger females, estrogen replacement therapy appears to be detrimental in older, postmenopausal females. In fact, epidemiological and experimental studies revealed that both age and estrogen replacement therapy increase the risk for stroke and the extent of brain injury following ischemic stroke in aged females (Liu *et al.*, 2009; Selvamani *et al.*, 2010). Recent studies in the systemic vasculature demonstrated that estrogen enhances the production of and reactivity to thromboxane (TXA_2) and other deleterious constrictor prostanoids in the female rat (Fulton *et al.*, 2002; Li *et al.*, 2005; Sellers *et al.*, 2008). Thus, it is important to determine if age exacerbates these deleterious effects of estrogen involving up-regulation of vascular TXA_2 production and enhancement of both vasoconstriction and hemostasis.

It is difficult to reconcile the apparent conflict in beneficial versus deleterious effects of estrogen on neurological and vascular function unless the effects of age are considered. Although the mechanisms underlying the beneficial effects of estrogen on cerebrovascular function have been studied extensively, the mechanisms responsible for age-dependent deleterious effects of estrogen are largely unknown. Furthermore, research focusing on the modulation of cerebrovascular reactivity with advancing age and estrogen replacement is extremely limited. This lack of understanding emphasizes

the importance of examining the cellular and molecular mechanisms underlying the role of age on the deleterious effects of estrogen replacement therapy and endogenous estrogen on the cerebral vasculature. Thus, in the present studies, the central hypothesis tested is that age enhances the deleterious effects of estrogen on the cerebrovasculature by augmenting the role of constrictor prostanoids in cerebrovascular reactivity and tone.

4.2. Methods

4.2.1. Ethical approval

All animal protocols were in accordance with "U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training" as detailed in the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" and approved by the Texas A&M University Institutional Animal Care and Use Committee.

4.2.2. Animals and maintenance

Neurological and vascular effects of estrogen, in both humans and experimental animals appear to depend upon age. Female rats of differing age groups, which approximate two key stages of "hormonal age" in humans, were studied. Thus, Sprague-Dawley rats of two different age groups were used in the present studies: mature multigravid adult (MA, 5-6 month, pre-menopausal, estrous cyclic), and reproductively senescent (RS, 12-14 month, post-menopausal, estrous acyclic) females (F). Age-matched male (M) rats were also studied as a control for the effects of aging. For the independent effects of age, it

was important to identify the phases of the estrous cycle in female experimental animals, and thus, daily vaginal smears were used to determine the stage of the estrous cycle at the time of experimentation. To eliminate the potential effects of cyclic surges in estrogen on vascular function, intact female rats were only used for experiments while in metestrus/diestrus phases. All rats were purchased from Harlan (Houston, TX) and housed at the main animal facility at Texas A&M University. Rats were housed in pairs, in standard plastic laboratory rat cages, in a well-ventilated room, maintained at constant temperature (21-26 °C), and controlled photoperiod (12 hour light: 12 hour dark). 16% protein global diet (soy and alfalfa-free to minimize dietary phytoestrogens, Harlan, Houston, TX) and water were provided *ad libitum*.

4.2.3. Plasma estrogen concentration

Female rats have a short estrous cycle which only lasts 4-5 days. Plasma estrogen concentrations oscillate markedly during the phases of the cycle, attaining a peak surge during proestrus and a nadir during metestrus. Because the proposed studies focus on the effects of estrogen levels and age, the endocrine status of all intact females was determined by vaginal smear. Smears were taken daily over 2-3 consecutive cycles immediately preceding and including the day of animal sacrifice and blood collection for measurement of estrogen. Estrous phase was determined by histological characteristics of the smears (LeFevre *et al.*, 1988; McLean *et al.*, 2012). Trunk blood was collected in EDTA coated tubes from all animals at the time of sacrifice, centrifuged, and the plasma

was stored at -80°C for later analysis of plasma 17β–estradiol levels by radioimmunoassay (RIA).

4.2.4. Pressurized cannulated MCA vessel preparation

Rats were humanely euthanized by rapid decapitation to avoid artifactual effects of anesthetics and minimize activation of neural and humoral pathways. The middle cerebral arteries (MCA) were isolated immediately and placed in chilled, Krebs-Henseleit-bicarbonate solution (KHB). The KHB was composed of (in mM) 118.0 NaCl, 25.0 $NaHCO_3$, 10.0 glucose, 4.74 KCl, 2.5 $CaCl_2$, 1.18 $MgSO_4$, and 1.18 KH_2PO_4. MCAs from each animal were cleaned of connective and brain tissue and arterial segments were prepared in triplicate. They were cannulated and tied securely to the pipettes using 11-0 ophthalmic suture. The glass micropipettes were filled with physiological salt solution (PSS) with albumin which contained the following (in mM): 145 NaCl, 4.7 KCl, 2.0 $CaCl_2$, 1.17 $MgSO_4$, 3.0 MOPS, 1.2 NaH_2PO_4, 5.0 glucose, 2.0 pyruvate, 0.02 EDTA , and 1% bovine serum albumin (BSA). The cannulated vessel was transferred to the stage of an inverted microscope (Olympus CKX41) equipped with a ×4 objective (numerical aperture of 0.13) and coupled with a video camera (Hitachi KP-M3AN), video monitor (Pelco PMM12A), DVD recorder (Phillips DVDR3475), and video micrometer (Colorado Video 307A). Both micropipettes were connected to a single reservoir system and were gradually adjusted to set the intraluminal pressure of the vessel at 85 mmHg without allowing flow through the vessel lumen. Leaks were detected by verifying that intraluminal diameter of the pressurized arteriole remained constant when the valve to the

reservoir system was closed. Only arterioles that were free of leaks were studied. The vessel chamber bath (Living Systems TC-09S) containing PSS+albumin was gradually warmed and maintained at 37 °C for the duration of the experiment. Luminal diameter was monitored continuously throughout the experiment. The vessels were allowed to equilibrate for 1 hour before being pretreated with pharmacological agents indicated below for 20 minutes. Cumulative concentration-response curves to arginine vasopressin (VP, 10^{-12} to 10^{-7} mol/L) were obtained by direct, cumulative additions of VP into the tissue baths, in the absence or presence of inhibitors including: (1) selective COX-1 inhibitor (SC560, 1 μM); or (2) selective COX-2 inhibitor (NS398, 10 μM). Diameter measurements were determined in response to cumulative concentrations of VP. Percent constriction was determined by the following equation: % constriction = $(B_D - B_x)/B_D$ *100, where B_D is the steady-state baseline diameter after inhibitor incubation and B_X is the diameter after each VP concentration. The concentration of VP that produced 50% of the maximal response (EC_{50}) was calculated individually from the log concentration-response curve of each MCA segment.

4.2.5. Prostanoid release assay (TXA_2 and PGI_2)

Vascular prostanoid production by the MCA was measured using incubation and radioimmunoassay methods adapted for microvessels, as described previously (Li *et al.*, 2008). Briefly, isolated MCA (3-4 mm axial length) were cleaned of connective tissue and fat, placed into chilled PSS without BSA (PSS-BSA) to rest for 60 minutes. The arterial segments were then transferred into 0.5 mL microcentrifuge tubes with 450 μL

chilled solution and gradually warmed in a water bath to 37°C for a 45 minute pre-incubation. The pre-incubation medium was carefully aspirated and 300 µL PSS-BSA alone (basal) or PSS-BSA with VP 10^{-9} M (low) or PSS-BSA with VP 10^{-7} M (high) was added and incubated at 37°C for 45 minutes. After incubation, the incubation media were collected and stored at -80°C until RIA of stable metabolites of PGI_2 (6-keto-prostaglandin $F_{1\alpha}$; 6-keto-$PGF_{1\alpha}$) and TXA_2 (TXB_2). MCA segments were saved and stored at -80°C for dry weight analysis.

4.2.6. Chemical reagents and drugs

The following reagents and drugs were used: 17β-estradiol (Innovative Research of America; Sarasota, FL), SC560 and NS398 (Cayman Chemical; Ann Arbor MI), arginine VP (Bachem; Torrance, CA). All other chemicals were purchased from Sigma Chemical (St. Louis, MO).

4.2.7. Statistics

All data are expressed as means ± SE; n indicates the number of animals studied. One- or two-way analysis of variance (ANOVAs) was used to detect significant differences among means of all experimental groups. If a main effect was identified, pairwise Student's t-tests were performed to detect significant differences between any two means of the data groups. Vascular function and prostanoid release data were analyzed using a two-way ANOVA for sex (M vs. F) and age (MA vs. RS). The effects of treatment (CTL, COX-1 inhibition, COX-2 inhibition) were analyzed in each experimental group using a

one-way ANOVA. Plasma estradiol levels, body weight and uterine weight were analyzed by sex and age using a two-way ANOVA and Student's t-tests. A P value ≤ 0.05 was considered significant.

4.3. Results

4.3.1. Effects of age and sex on estrogen levels, body weight, and uterine weight

Plasma 17β-estradiol concentrations, body weight and uterine weight are summarized in Table 3. Due to the design of the experiments, the younger MA females were in metestrus or diestrus phase of the estrous cycle (as determined by vaginal smears) and thus were sacrificed during low, non-surge, estradiol levels. Previous studies reported that random estradiol levels of young intact cycling females average 43.9 ± 13. (Li *et al.*, 2005). Uterine weights did not significantly differ with age ($P > 0.05$). Body weight did not differ with age in females; however, RS males were significantly heavier than MA males ($P \leq 0.01$). Body weights were significantly different between males and females in both young MA and older RS rats ($P \leq 0.01$).

Table 3. Plasma 17β-estradiol concentrations, body weight and uterine weight of mature multigravid.

Group	Plasma Estradiol (pg/mL)	Body Weight (g)	Uterine Weight (g/100g body weight)
	n=10-14	n=14-15	n=14
MAF	3.24 ± 1.38^{b}	303 ± 5.8^{a}	0.41 ± 0.04^{a}
MAM	2.55 ± 1.06^{b}	491 ± 10.9^{b}	
RSF	6.59 ± 1.32^{c}	305 ± 9.5^{a}	0.34 ± 0.03^{a}
RSM	0.33 ± 0.09^{a}	559 ± 12.3^{c}	

Mature multigravid adult (MA, 4-6 mo.) female (F) (MAF) or age-matched male (M) rats (MAM), and reproductively senescent (RS, 10-12 mo.) female (F) (RSF) or age matched male rats (RSF). Values are means ± SE; n=no of animals studied. $^{a\text{-}c}$ $0.0001 \leq P \leq 0.01$, values within columns (estradiol, body weight, uterine weight) with different superscripts are significantly different.

4.3.2. Effects of age and sex on vascular reactivity to VP

The effects of age and sex on vasopressin-induced vasoconstriction of MCA are shown in Figure 13, Figure 14 and Table 4. Comparison of control curves (Figure 13) revealed significant age differences in both male and female at the middle VP concentration. At the maximal VP concentration, age had a significant effect in females but not in males, and in older RS rats sex also had a significant effect. In older RSF rats, VP-stimulated constriction was significantly decreased at both middle and maximal VP concentrations as compared with younger MAF. In both MA and RS male rats, VP-induced constriction did not differ significantly from MAF. At the middle VP concentration constrictions in RSM were significantly attenuated compared to MAF and MAM; however, sensitivity to VP did not differ.

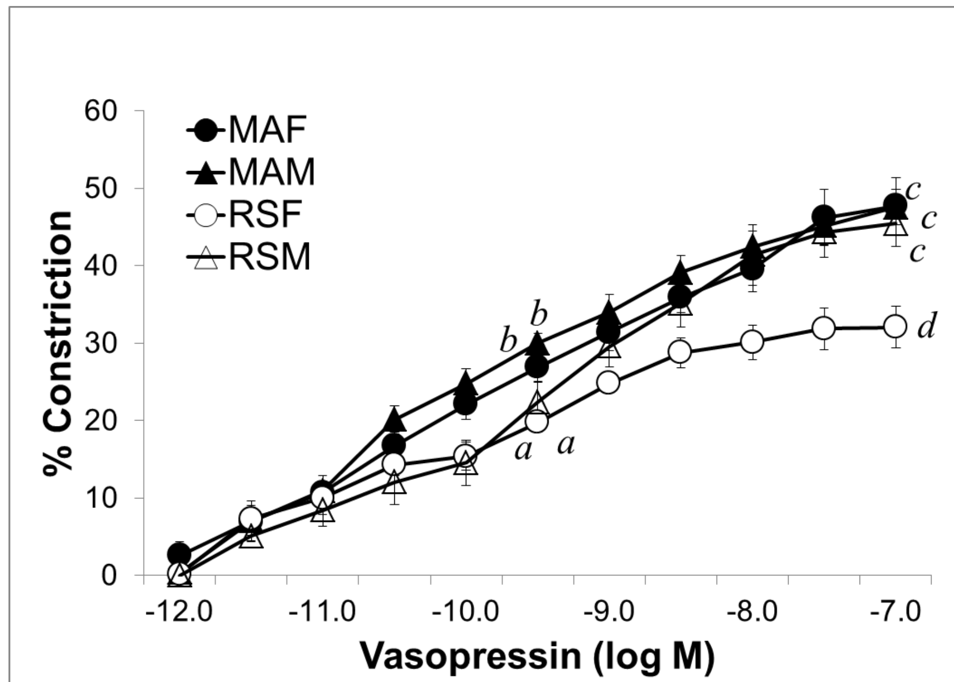

Figure 13. Concentration-response curves for vasopressin (VP) in endothelium-intact pressurized middle cerebral artery segments prepared from MAF, MAM, RSF, and RSM.
Mature multigravid adult (MA, 4-6 mo.) female (MAF) or age-matched male rats (MAM), and reproductively senescent rats (RS, 10-12 mo.) female (RSF) or age-matched male rats (RSM). Data points represent means ± SE (n=6 rats/group).[a-d]$0.0006 \leq P \leq 0.04$, mean values without common superscript differ significantly at middle and maximal concentrations of VP.

In MAF, VP produced concentration-dependent constrictions with a maximal concentration response of 47.7% and an EC_{50} of 0.39 nM (Figure 14A). Both COX-1 and COX-2 selective inhibitors, SC560 and NS398, significantly attenuated constriction at middle and maximal VP concentrations. Compared with the control group, maximal constriction was reduced by 58% and 29% by SC560 and NS398 respectively.

Maximal response to VP was 32.1% in RSF with an EC_{50} of 0.13 nM (Figure 14C). Sensitivity to VP (EC_{50}) did not differ with age in MAF vs. RSF. SC560 significantly

enhanced maximal constriction to VP in RSF by 39%, yet NS398 had no significant effect. It is important to note that the reduction in VP constriction in RSF (as compared to MAF, MAM and RSM) was due to an enhancement in COX-1 derived dilator prostanoids.

In MAM, VP produced concentration-dependent constrictions with a maximal concentration response of 47.6% and an EC_{50} of 0.10 nM (Figure 14B). At the middle VP concentration both SC560 and NS398 attenuated constriction in MAM; however, these differences dissipated at the maximal VP concentration.

In RSM, VP produced concentration-dependent constrictions with a maximal concentration response of 45.5% and an EC_{50} of 0.50 nM (Figure 14D). SC560 and NS398 had no significant effect on VP constriction in older RSM rats at either middle or maximal VP concentrations.

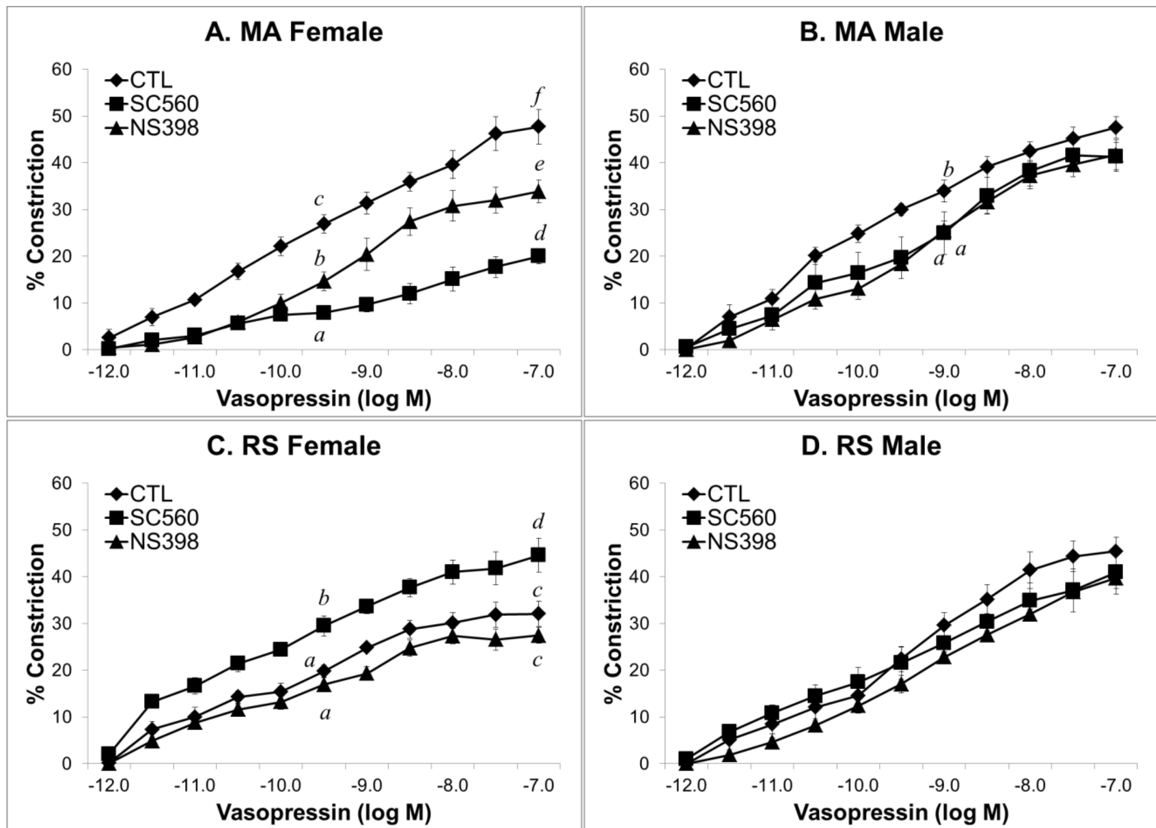

Figure 14. Concentration-response curves for VP in endothelium-intact pressurized middle cerebral artery segments prepared from male and female Sprague-Dawley rats in the presence of selective COX inhibitors SC560 (COX-1), NS398 (COX-2), or vehicle control.
Preparations were studied in triplicate from each animal group: mature multigravid adult (MA, 4-6 mo.) female (MAF) **(A)**; or age-matched male rats (MAM) **(B);** reproductively senescent (RS, 10-12 mo.) female (RSF) **(C);** or age-matched male rats (RSM) **(D)**. Data points represent means ± SE (n=6 rats/group). $^{a\text{-}f}0.0001 \leq P \leq 0.02$, mean values without common superscript differ significantly at middle and maximal concentrations of VP. In RSM, there were no significant differences among CTL, SC560 and NS398 groups.

Table 4. Middle- and Maximal-VP concentration and EC_{50}.

Group	Middle VP concentration ($10^{-9.5}$ M, % constriction)	Maximal VP concentration (10^{-7} M, % constriction)	EC_{50} (nM)
Control			
MAF	26.9 ± 2.0^{b}	47.7 ± 3.7^{b}	0.39 ± 0.21^{a}
MAM	30.0 ± 1.3^{b}	47.6 ± 2.3^{b}	0.10 ± 0.03^{a}
RSF	19.8 ± 1.3^{a}	32.1 ± 2.7^{a}	0.13 ± 0.06^{a}
RSM	22.4 ± 2.7^{a}	45.5 ± 3.0^{b}	0.50 ± 0.25^{a}
SC560			
MAF	7.9 ± 1.1^{a}	20.0 ± 1.6^{a}	3.77 ± 2.94^{a}
MAM	19.7 ± 4.5^{b}	41.3 ± 3.1^{b}	0.72 ± 0.36^{a}
RSF	29.5 ± 2.2^{c}	44.6 ± 3.7^{b}	0.06 ± 0.02^{a}
RSM	21.5 ± 3.5^{b}	41.0 ± 4.6^{b}	0.31 ± 0.16^{a}
NS398			
MAF	14.6 ± 2.0^{a}	33.9 ± 2.4^{a}	0.81 ± 0.32^{ab}
MAM	18.4 ± 1.3^{a}	41.7 ± 3.2^{b}	0.38 ± 0.10^{b}
RSF	17.0 ± 1.5^{a}	27.5 ± 1.7^{a}	0.21 ± 0.07^{a}
RSM	17.1 ± 1.9^{a}	39.7 ± 2.3^{b}	0.74 ± 0.24^{b}

Mature multigravid adult (MA, 4-6 mo.) female (F) (MAF) or age-matched male (M) rats (MAM), and reproductively senescent (RS, 10-12 mo.) female (F) (RSF) or age matched male rats (RSF). Values are means ± SE; n=no of animals studied. $^{a\text{-}c}0.0001 \leq P \leq 0.02$, values within columns (middle VP concentration, maximal VP concentration, EC_{50}) with different superscripts are significantly different.

4.3.3. Effects of age and sex on basal and VP-stimulated PGI_2 release

Basal and VP-stimulated (low concentration 10^{-9} M; high concentration 10^{-7} M) release of 6-keto-$PGF_{1\alpha}$ are shown in Figure 15 and Table 5. Basal and low concentration-VP stimulated release of 6-keto-$PGF_{1\alpha}$ did not significantly differ between groups (MAF, MAM, RSF, or RSM) ($P \leq 0.05$). Within each groups VP increased 6-keto-$PGF_{1\alpha}$ production in a concentration-dependent manner. Low-concentration VP-stimulated 6-

keto-$PGF_{1\alpha}$ release did not differ significantly between groups. However low-VP increased 6-keto $PGF_{1\alpha}$ by fourfold in RSF and fivefold in MAF, MAM and RSM from their respective basal levels. High-concentration VP increased 6-keto-$PGF_{1\alpha}$ production eightfold in MAF, sevenfold in MAM, and sixfold in RSF and RSM from their respective basal levels. At high concentration VP MA females produced significantly more 6-keto $PGF_{1\alpha}$ than RS females and all other groups.

Table 5. Basal, low- and high-VP stimulated PGI_2 production.

Group	Basal (pg/mg/45 min)	Low VP ($10^{-9)}$ (pg/mg/45 min)	High VP (10^{-7}) (pg/mg/45 min)
MAF	5,349±1,170[a]	26,207±5,245[a]	40,199±5,920[b]
MAM	4,126±767.8[a]	18,814±4,651[a]	28,837±3,817[ab]
RSF	3,504±752.2[a]	13,973±2,414[a]	22,302±4,611[a]
RSM	3,985±743.1[a]	19,329±2,359[a]	24,972±2,126[ab]

Mature multigravid adult (MA, 4-6 mo.) female (F) (MAF) or age-matched male (M) rats (MAM), and reproductively senescent (RS, 10-12 mo.) female (F) (RSF) or age matched male rats (RSF). Values are means ± SE; n=no of animals studied. [a-c]$0.0001 \leq P \leq 0.02$, values within columns (Basal, low-VP, and high-VP) with different superscripts are significantly different.

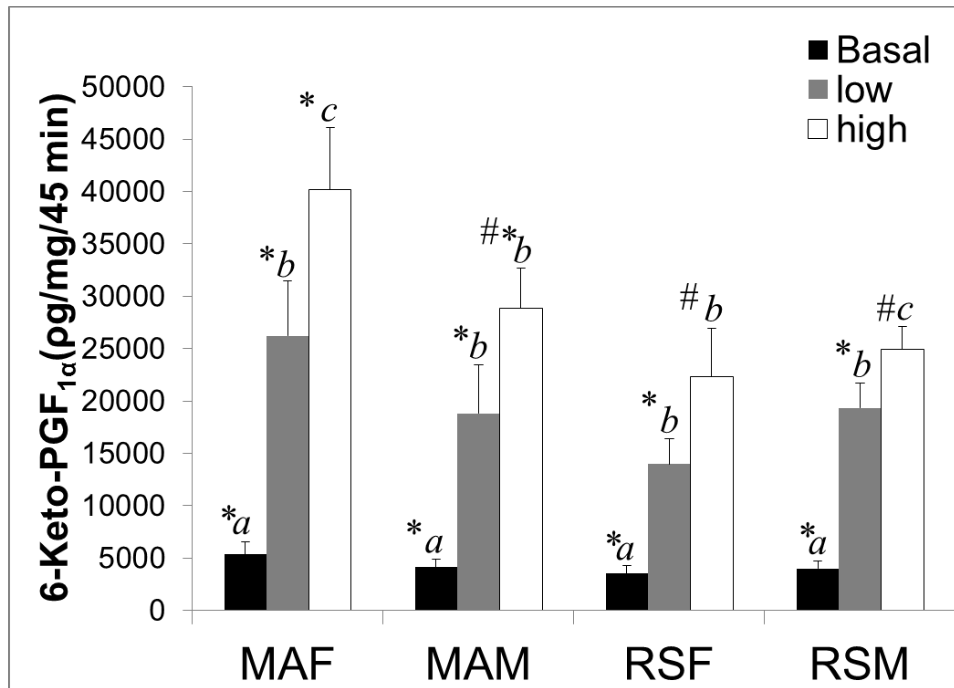

Figure 15. Basal and VP-stimulated (low concentration, 10^{-9} M; high concentration 10^{-7} M) release of 6-keto-$PGF_{1\alpha}$ by middle cerebral artery segments from MAF, MAM, RSF and RSM rats. Mature multigravid adult (MA, 4-6 mo.) female (MAF) or age-matched male rats (MAM), and reproductively senescent (RS, 10-12 mo.) female (RSF) or age-matched male rats (RSM). Data points represent means ± SE (n=6 rats/group). $^{a\text{-}c}P \leq 0.0001$, mean values within groups (MAF, MAM, RSF, RSM) without common superscript are significantly different.$^{*,\#,+}0.0001 \leq P \leq 0.02$ mean values between groups (MAF vs. MAM vs. RSF vs. RSM) with different superscripts are significantly different.

4.3.4. Effects of age and sex on basal and VP-stimulated TXA_2 release

Basal and vasopressin-stimulated (low concentration 10^{-9} M; or high concentration 10^{-7} M) release of TXB_2 are shown in Figure 16 and Table 6. Basal and low concentration-VP stimulated release of TXB_2 did not significantly differ between groups (MAF, MAM, RSF, or RSM). Within all groups VP increased TXB_2 production in a concentration-dependent manner. Low concentration-VP increased TXB_2 production twofold in MAF, RSF and MAM, and threefold in RSM from their respective basal

levels. High-VP concentration increased 6-keto-$PGF_{1\alpha}$ production sixfold in MAF, threefold in MAM, and fourfold in RSF and RSM from their respective basal levels. At high concentration-VP, MAF produced significantly more TXB_2 than all other groups.

Table 6. Basal, low- and high-VP stimulated TXA_2 production.

Group	Basal (pg/mg/45 min)	Low VP ($10^{-9)}$ (pg/mg/45 min)	High VP (10^{-7}) (pg/mg/45 min)
MAF	285.7±44.6[a]	542.3±43.2[a]	1,663±118.7[b]
MAM	338.0±38.7[a]	641.5±130.6[a]	1,063±139.0[a]
RSF	264.9±46.7[a]	487.4±66.0[a]	1,000±110.9
RSM	263.6±42.7[a]	692.7±87.5[a]	1,168±105.4[a]

Mature multigravid adult (MA, 4-6 mo.) female (F) (MAF) or age-matched male (M) rats (MAM), and reproductively senescent (RS, 10-12 mo.) female (F) (RSF) or age matched male rats (RSF). Values are means ± SE; n=no of animals studied. [a-c]$0.0001 \leq P \leq 0.003$, values within columns (basal, low-VP and high-VP) with different superscripts are significantly different.

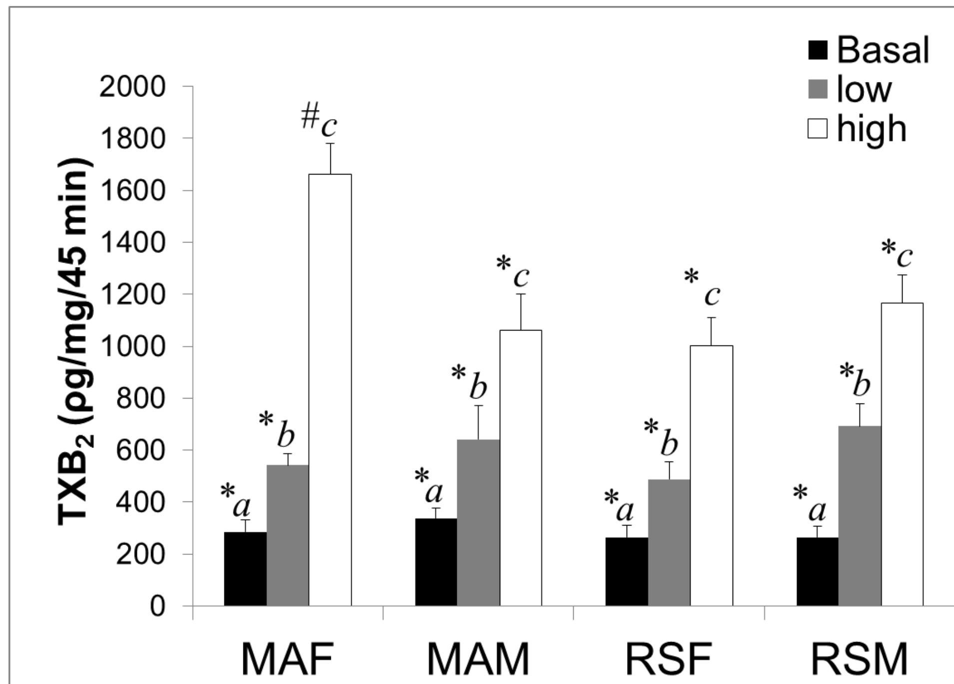

Figure 16. Basal and VP-stimulated (low concentration, 10^{-9} M; or high concentration 10^{-7} M) release of TXB_2 by middle cerebral artery segments from MAF, MAM, RSF and RSM rats. Mature multigravid adult (MA, 4-6 mo.) female (MAF) or age-matched male rats (MAM), and reproductively senescent (RS, 10-12 mo.) female (RSF) or age-matched male rats (RSM). Data points represent means ± SE (n=6 rats/group). $^{a\text{-}c}$0.0001≤*P*≤0.003, mean values within groups (MAF, MAM, RSF, RSM) without common superscript are significantly different.$^{*, \#, +}$ 0.0001≤*P*≤0.003 mean values between groups (MAF vs. MAM vs. RSF vs. RSM) with different superscripts are significantly different.

4.4. Discussion

The present study revealed the novel finding that age and sex alter cerebrovascular reactivity and prostanoid production in the MCA of Sprague-Dawley rats. This is the first study to examine the effects of age and sex on the mechanisms underlying cerebrovascular reactivity to VP. The main findings of the study are that: 1) VP-mediated constriction is altered by age in intact females but is unchanged in male rats, 2) selective blockade of COX-1 or COX-2 produced age-dependent changes in

cerebrovascular reactivity to VP, and 3) VP-stimulated PGI_2 and TXA_2 production are enhanced by endogenous estrogen. In intact males there were no changes in VP-induced vasoconstriction. Additionally, there were no significant differences in basal, low- or high-VP stimulated PGI_2 or TXA_2 production as measured by the stable metabolites 6-keto-$PGF_{1\alpha}$ or TXB_2 of young MA or older RS males. In contrast, there were marked differences in cerebrovascular reactivity and prostanoid release with advancing age in females. Older intact females in this study were acyclic and thus had constant low endogenous estrogen levels. These older RS females exhibited reduced maximal constrictor responses to VP, which can be attributed to enhanced COX-1 dependent dilator prostanoids. VP-induced vasoconstriction in younger MA females (which were normally cycling) utilized both COX-1 and COX-2 constrictor prostanoids. Additionally, high concentration VP-stimulated PGI_2 and TXA_2 production were enhanced by endogenous estrogen and decreased with advancing age in intact female but not male rats.

4.4.1. Effect of age on mechanisms of cerebrovascular function

Endothelial dysfunction increases in men after age 40 and in women after age 55 (Celermajer *et al.*, 1994). While the exact cause of the decline in endothelial function is unknown, aging is usually associated with a reduction in the ability to elicit endothelium-dependent vasodilation in both animals and in humans (Csiszar *et al.*, 2002; Taddei *et al.*, 2001; Tschudi *et al.*, 1996). This loss of function occurs via numerous mechanisms leading to attenuated nitric oxide-mediated dilation (Csiszar *et al.*, 2002;

Kloss *et al.*, 2000; Taddei *et al.*, 2001; Tschudi *et al.*, 1996). In addition to the age-related changes in nitric oxide, an enhancement of vasoconstrictor prostaglandins further potentiates age-dependent endothelial dysfunction. With advancing age, both COX-1 and COX-2 expression are upregulated by oxidative stress, whereas PGI_2 receptor (IP) expression decreases (Ge *et al.*, 1995; Numaguchi *et al.*, 1999; Shi *et al.*, 2008; Tang *et al.*, 2008). There is also indirect evidence suggesting that untransformed PGH_2 (which interacts with the TXA_2 (TP) receptor) is also augmented with aging due to COX-1/COX-2 upregulation (Dai *et al.*, 1992; Hynes *et al.*, 1987; Lin *et al.*, 1994; Pagano *et al.*, 1991). Numerous studies have reported increases in TXA_2 and TXS mRNA in aorta and mesenteric arteries with age (Matz *et al.*, 2000; Tang *et al.*, 2008). Thus, in agreement with this study, the balance of dilator to constrictor prostanoids appears to be altered with age, by decreased release and/or reactivity to PGI_2 and augmentation of constrictor prostanoid PGH_2 and TXA_2 production.

4.4.2. Effects of sex and estrogen on mechanisms of cerebrovascular function

Women tend to exhibit higher levels of cerebral blood flow (CBF) than men when they are younger but this difference becomes less significant later in life around the onset of menopause (Rodriguez *et al.*, 1988; Shaw *et al.*, 1984). Additionally CBF varies throughout the menstrual cycle (Brackley *et al.*, 1999; Diomedi *et al.*, 2001) and is altered during pregnancy (Brackley *et al.*, 1998). Thus, chronic exposure to estrogen positively alters cerebrovascular function. Interestingly there appear to be striking sex-differences in the modulation of cerebrovascular arterial tone, with male arteries

exhibiting greater myogenic tone in response to increasing pressure as compared to females (Geary *et al.*, 1998). Numerous studies have reported that estrogen enhances the production and/or the sensitivity of cerebral arteries to vasodilatory factors nitric oxide and PGI_2 (Geary *et al.*, 2000a; Geary *et al.*, 1998; Ospina *et al.*, 2003; Ospina *et al.*, 2002; Pelligrino *et al.*, 2000; Skarsgard *et al.*, 1997). In the cerebrovasculature, estrogen appears to shift the prostaglandin balance towards greater production of vasodilator prostanoids (Ospina *et al.*, 2003). In cerebral vessels, estrogen elevates both COX-1 and PGIS resulting in enhanced PGI_2 production (Geary *et al.*, 2000a; Ospina *et al.*, 2003; Ospina *et al.*, 2002) in young rats. Interestingly, TXA_2 production was also slightly yet significantly elevated in young animals with estrogen-treatment, perhaps due to increased COX-1 levels (Lin *et al.*, 2002; Ospina *et al.*, 2002). The age-and sex-dependent shifts in dilator and constrictor prostanoids observed in the present study reveal important novel roles for the prostanoids in cerebrovascular function during aging.

4.4.3. Divergent effects of estrogen and age on cerebrovascular function: beneficial and deleterious

Earlier findings in human epidemiological (Kawas *et al.*, 1997; Levy *et al.*, 1988; Messerli *et al.*, 1987) and experimental animal studies (Farhat *et al.*, 1996; Karas, 2002; Simpkins *et al.*, 1997) led to the belief that estrogen replacement therapy exerts beneficial effects on neurological and cardiovascular health and is protective against diseases such as dementia, coronary artery disease, hypertension, and stroke. An

abundance of evidence from experimental animal studies has established that estrogen does exert beneficial or protective effects on the cerebrovasculature by reducing vascular reactivity and thereby increasing blood flow through nitric oxide- and vasodilator prostanoid-dependent mechanisms (Geary *et al.*, 2000a; Geary *et al.*, 1998; McNeill *et al.*, 1999; McNeill *et al.*, 2002; Orshal *et al.*, 2004; Osanai *et al.*, 2000; Ospina *et al.*, 2003; Ospina *et al.*, 2002). In contrast, more recent human epidemiological findings such as the HERS (Hulley *et al.*, 1998), HERSII (Grady *et al.*, 2000; Hulley *et al.*, 2002), and WHI studies (Hendrix *et al.*, 2006; Wassertheil-Smoller *et al.*, 2003) all suggest that in older women estrogen replacement therapy increases the incidences of neurological (dementia and stroke) and vascular diseases (coronary artery disease, hypertension, and venous thrombosis). Thus, the role of estrogen in cardiovascular health and disease has become controversial. Indeed, recent studies of the systemic vasculature have clearly established that estrogen exerts deleterious effects on the female vasculature through upregulation of COX-2, TXS, and TP receptor expression, thereby enhancing release of and responsiveness to constrictor prostanoids in the female systemic vasculature. Although numerous studies have examined the beneficial effects of estrogen replacement in numerous vascular beds of young animals (Geary *et al.*, 2000a; Li *et al.*, 2008; Ospina *et al.*, 2003), the present study is the first to examine the divergent effects of estrogen supplementation on cerebrovascular reactivity and prostanoid production in male and female rats at both younger and advanced age.

The results of this study provide important and novel new information on the effects of estrogen and advancing age on cerebrovascular function. Further understanding of the mechanisms by which estrogen exerts its beneficial and detrimental effects on the cerebrovasculature will perhaps lead to new age-and sex-specific therapeutic agents designed specifically to target the cerebrovasculature and other estrogen-responsive tissues.

5. EFFECTS OF AGE AND ESTROGEN ON CEREBROVASCULAR FUNCTION IN THE RAT MIDDLE CEREBRAL ARTERY: THE SHIFT FROM BENEFICIAL TO DETRIMENTAL EFFECTS

5.1. Introduction

The risk of cardiovascular diseases and stroke increases with advancing age; yet most research utilizes young animals. The beneficial effects of estrogen on the systemic and cerebral circulations are well documented in numerous animal studies; however, human clinical trials have reported increased incidences and severity of stroke in women undergoing hormone replacement therapy with estrogen. Failure to account for age-related changes in the mechanisms that modulate vascular tone may help explain the paradox between the beneficial effects of estrogen observed in animal studies and the deleterious effects of estrogen reported in human clinical trials. Therefore, it is important to determine if age exacerbates the deleterious effects of estrogen on cerebrovascular function in female rats.

Recent studies reported that age and sex alter cerebrovascular reactivity in intact Sprague-Dawley rats (Chapter 4). The roles of COX-1 and COX-2 derived prostanoids in modulating vasopressin-induced vasoconstriction of the middle cerebral artery (MCA) were altered with age in female but not in male rats (Chapter 4). Additionally, the production of prostacyclin (PGI_2) and thromboxane (TXA_2) by the MCA were altered with age in female rats (Chapter 4). Thus, in the present study the combined effects of

age and estrogen on the modulation of cerebrovascular function were examined in sexually mature and reproductively senescent female rats. The present studies tested the central hypothesis that age enhances the deleterious effects of exogenous estrogen on the cerebrovasculature by enhancing the role of constrictor prostanoids in potentiating female cerebrovascular reactivity. These studies appear to be the first to examine the combined effects of age and estrogen on the vasoconstrictor reactivity to vasopressin in the female cerebrovascular circulation. The aims of the present study were to determine the effects of age and estrogen on: 1) vascular reactivity of the female MCA to vasopressin, a systemic vasoconstrictor hormone important in the regulation in systemic and cerebrovascular function and 2) basal and agonist-stimulated production of the primary dilator and constrictor prostanoids, PGI_2 and TXA_2, respectively, that modulate cerebrovascular reactivity to VP in the female MCA.

5.2. Methods

5.2.1. Ethical approval

All animal protocols were in accordance with "U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training" as detailed in the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" and approved by Texas A&M University Institutional Animal Care and Use Committee.

5.2.2. Animals and maintenance

Female rats of differing age groups approximating the key stages of "hormonal age" in humans were studied: mature multigravid adults (MA, 5-6 month, pre-menopausal, estrous cyclic), and reproductively senescent adults (RS, 12-14 month, post-menopausal, estrous acyclic). Rats were purchased from Harlan (Houston, TX) and housed at the main animal facility at Texas A&M University. Rats were housed in pairs, in standard plastic laboratory rat cages, in a well-ventilated room, maintained at constant temperature (21-26 °C), with controlled photoperiod (12 hour light: 12 hour dark). 16% protein global diet (soy and alfalfa-free to minimize dietary phytoestrogens, Harlan, Houston, TX) and water were provided *ad libitum*.

5.2.3. Ovariectomy and estrogen replacement therapy

All female rats underwent bilateral ovariectomy (O) using standard surgical methods. Briefly, a dorsomedial incision through the skin was made. Subcutaneous fascia was cleaned and 1 cm bilateral dorsolateral incisions made in the abdominal wall to expose the ovarian fat pad. The ovary and uterus were carefully externalized, ligated and then transected. The ligated uterine horn was swabbed with Betadyne and allowed to retract into the abdominal cavity. The abdominal wall was closed with 4-0 vicryl absorbable suture and the skin incision approximated using wound clips. Prior to anesthetic recovery, estrogen (E) replacement therapy was initiated by subcutaneous implantation of three 0.05 mg 17β-estradiol 60-day time release pellets (Innovative Research, Sarasota, FL). Rats not receiving estrogen replacement were implanted with vehicle-

control placebo pellets. All rats were sacrificed for in vitro experiments 14-21 days post-surgery.

5.2.4. Pressurized cannulated MCA vessel preparation

Rats were humanely euthanized by rapid decapitation to avoid artifactual effects of anesthetics and minimize activation of neural and humoral pathways. The middle cerebral arteries (MCA) were isolated immediately and placed in chilled, Krebs-Henseleit-bicarbonate solution (KHB). The KHB was composed of (in mM) 118.0 NaCl, 25.0 $NaHCO_3$, 10.0 glucose, 4.74 KCl, 2.5 $CaCl_2$, 1.18 $MgSO_4$, and 1.18 KH_2PO_4. MCAs from each animal were cleaned of connective and brain tissue and arterial segments were prepared in triplicate. They were cannulated and tied securely to the pipettes using 11-0 ophthalmic suture. The glass micropipettes were filled with physiological salt solution (PSS) with albumin which contained the following (in mM): 145 NaCl, 4.7 KCl, 2.0 $CaCl_2$, 1.17 $MgSO_4$, 3.0 MOPS, 1.2 NaH_2PO_4, 5.0 glucose, 2.0 pyruvate, 0.02 EDTA , and 1% bovine serum albumin (BSA). The cannulated vessel was transferred to the stage of an inverted microscope (Olympus CKX41) equipped with a ×4 objective (numerical aperture of 0.13) and coupled with a video camera (Hitachi KP-M3AN), video monitor (Pelco PMM12A), DVD recorder (Phillips DVDR3475), and video micrometer (Colorado Video 307A). Both micropipettes were connected to a single reservoir system and were gradually adjusted to set the intraluminal pressure of the vessel at 85 mmHg without allowing flow through the vessel lumen. Leaks were detected by verifying that intraluminal diameter of the pressurized arteriole remained constant when the valve to the

reservoir system was closed. Only arterioles that were free of leaks were studied. The vessel chamber bath (Living Systems TC-09S) containing PSS+albumin was gradually warmed and maintained at 37 °C for the duration of the experiment. Luminal diameter was monitored continuously throughout the experiment. The vessels were allowed to equilibrate for 1 hour before being pretreated with pharmacological agents indicated below for 20 minutes. Cumulative concentration-response curves to arginine vasopressin (VP, 10^{-12} to 10^{-7} mol/L) were obtained by direct, cumulative additions of VP into the tissue baths, in the absence or presence of inhibitors including: (1) selective COX-1 inhibitor (SC560, 1 μM); or (2) selective COX-2 inhibitor (NS398, 10 μM). Diameter measurements were determined in response to cumulative concentrations of VP. Percent constriction was determined by the following equation: % constriction = $(B_D - B_x)/B_D$ *100, where B_D is the steady-state baseline diameter after inhibitor incubation and B_X is the diameter after each VP concentration. The concentration of VP that produced 50% of the maximal response (EC_{50}) was calculated individually from the log concentration-response curve of each MCA segment.

5.2.5. Prostanoid release assay (TXA_2 and PGI_2)

Vascular prostanoid production by the MCA was measured using incubation and radioimmunoassay methods adapted for microvessels, as described previously (Li *et al.*, 2008). Briefly, isolated MCA (3-4 mm axial length) were cleaned of all connective tissue and fat, placed into chilled PSS without BSA (PSS-BSA) to rest for 60 minutes. The arteries were then transferred into 0.5 mL microcentrifuge tubes with 450 μL chilled

solution and gradually warmed in a water bath to 37°C for a 45 minute pre-incubation. The pre-incubation medium was carefully aspirated and 300 µL PSS-BSA alone (basal) or PSS-BSA with VP 10^{-9} M (low) or PSS-BSA with VP 10^{-7} M (high) was added and incubated at 37°C for 45 minutes. After incubation, the incubation media were collected and stored at -80°C until RIA of stable metabolites of PGI_2 (6-keto-prostaglandin $F_{1\alpha}$; 6-keto-$PGF_{1\alpha}$) and TXA_2 (TXB_2). MCA segments were saved and stored at -80°C for dry weight analysis.

5.2.6. Chemical reagents and drugs

The following reagents and drugs were used: 17β-estradiol (Innovative Research of America; Sarasota, FL), SC560 and NS398 (Cayman Chemical; Ann Arbor MI), arginine VP (Bachem; Torrance, CA). All other chemicals were purchased from Sigma Chemical (St. Louis, MO).

5.2.7. Statistics

All data are expressed as means ± SE; n indicates the number of animals studied. One- or two-way analysis of variance (ANOVAs) was used to detect significant differences among means of all experimental groups. If a main effect was identified, pairwise Student's t-tests were performed to detect significant differences between any two mean of the data groups. A P value ≤ 0.05 was considered significant. Vascular function and prostanoid release data were analyzed using a two-way ANOVA for estrogen (O vs. E) and age (MA vs. RS). The effects of treatment (CTL, COX-1 inhibition, COX-2

inhibition) were analyzed in each experimental group using a one-way ANOVA. Plasma estradiol levels, body weight and uterine weight were analyzed by estrogen and age using a two-way ANOVA and Student's t-tests.

5.3. Results

5.3.1. Effects of age and sex on estrogen levels, body weight, and uterine weight

Plasma 17β-estradiol concentrations, body weight and uterine weight are summarized in Table 7. Both younger MA and older RS females that were ovariectomized and given estrogen replacement had significantly lower body weights and significantly greater uterine weights as compared to ovariectomized females of the same age. Estradiol levels followed the same trends, in MAO and RSO ovariectomy decreased estradiol levels and estrogen replacement significantly increased estradiol levels in MAE and RSE.

Table 7. Plasma 17β-estradiol concentrations, body weight and uterine weight of MAO, MAE, RSO and RSE.

Group	Estradiol (pg/mL) n=13-15	Body Weight (g) n=13-15	Uterine Weight (g/100g body weight) n=12-14
MAO	1.9 ± 1.3^{a}	308.9 ± 12.9^{b}	0.14 ± 0.03^{a}
MAE	33.6 ± 7.3^{b}	259.9 ± 6.9^{a}	0.24 ± 0.02^{b}
RSO	0.2 ± 0.1^{a}	339.6 ± 4.1^{c}	0.10 ± 0.02^{a}
RSE	37.4 ± 8.4^{b}	302.8 ± 7.0^{b}	0.29 ± 0.03^{b}

Mature multigravid adult female rats (MA, 4-6 mo.), either ovariectomized (MAO) or ovariectomized and estrogen-replaced (MAE), and reproductively senescent female rats (RS, 10-12 mo.), either ovariectomized (RSO) or ovariectomized and estrogen-replaced (RSE). Values are means ± SE; n=no of animals studied. $^{a\text{-}c}0.0001 \leq P \leq 0.04$, values within columns (estradiol, body weight, uterine weight) with different superscripts are significantly different.

5.3.2. Effects of age and sex on vascular reactivity to VP

The effects of age and sex on vasopressin-induced constriction are shown in Figure 17, Figure 18, and Table 8. Comparison of the concentration-response control curves in each of the four experimental groups (MAO, MAE, RSO, RSE) revealed significant age- and estrogen-dependent differences at both middle- and maximal-VP concentration (Figure 17).

In older RSO rats, VP-stimulated constriction was significantly decreased at mid-VP concentration and sensitivity to VP was also significantly decreased in RSO as compared to MAO; yet, there was no difference in max-VP concentration constriction between MAO and RSO. In contrast, maximal constriction to VP was augmented significantly (50.6%) with estrogen in older RSE females as compared to younger MAE females. Estrogen attenuated constriction by 21% in younger MAE females as compared to ovariectomized MAO females, however, sensitivity to VP (EC_{50}) was not altered (MAO vs. MAE). In contrast, estrogen augmented constriction by 27% in older RSE females as compared to RSO and additionally estrogen therapy significantly increased sensitivity to VP in older RS female.

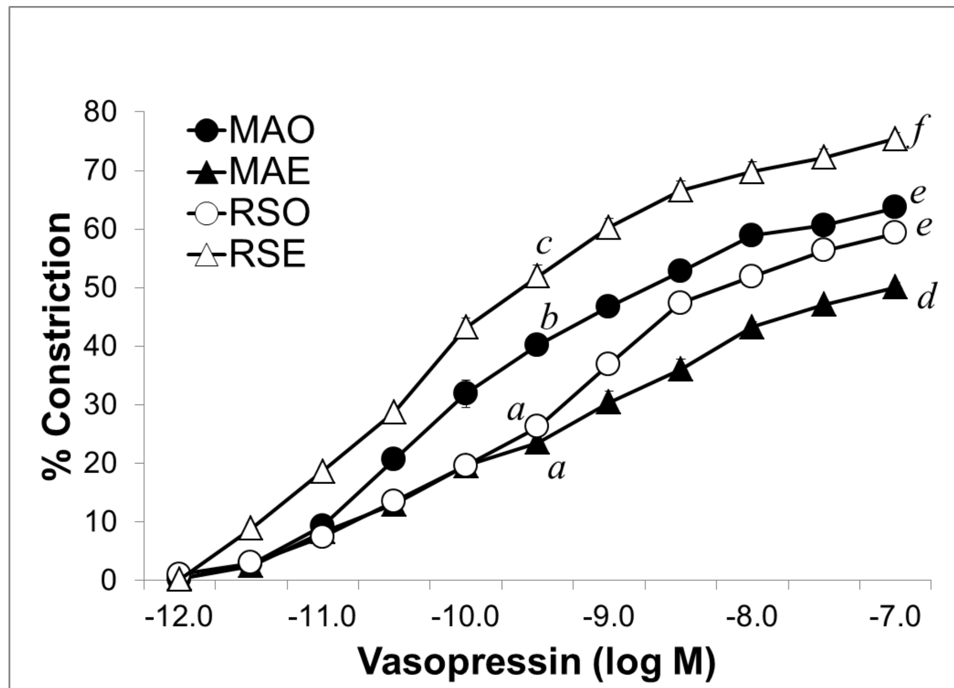

Figure 17. Concentration-response curves for vasopressin (VP) in endothelium-intact pressurized middle cerebral artery segments prepared from MAO, MAE, RSO, and RSE.
Mature multigravid adult female rats (MA, 4-6 mo.), either ovariectomized (MAO) or ovariectomized and estrogen-replaced (MAE), and reproductively senescent female rats (RS, 10-12 mo.), either ovariectomized (RSO) or ovariectomized and estrogen-replaced (RSE). Data points represent means ± SE (n=6-7 rats/group).[a-f]$0.0001 \leq P \leq 0.009$, mean values without common superscript differ significantly at middle and maximal concentrations of VP.

In MAO, VP produced concentration-dependent constrictions with a maximal response of 63.6% and an EC_{50} of 0.14 nM. Both COX-1 and COX-2 selective inhibitors, SC560 and NS398, significantly attenuated vasoconstriction at both middle and maximal concentrations of VP (Figure 18A). Compared with the control MAO group, maximal constriction was reduced by 14% and 16% by SC560 and NS398, respectively.

Maximal VP response was 59.3% in RSO with an EC_{50} of 0.40 nM (Figure 18C). Similar to MAO, in RSO, SC560 and NS398 reduced maximal-VP constriction in the same way, 23% and 26 % respectively.

In MAE, VP produced concentration-dependent constrictions with a maximal response of 50.07% and an EC_{50} of 0.40 nM (Figure 18B). Once again both COX-1 and COX-2 selective inhibitors, SC560 and NS398, significantly attenuated constriction at middle a maximal VP concentrations. Compared with the control MAE group, maximal constriction was reduced to a greater extent by SC560 than by NS398 (59% vs. 34%) indicating a greater role for COX-1 constrictor prostanoids in MAE.

Maximal VP response was 75.42% in RSE with an EC_{50} of 0.07 nM (Figure 18D). Both COX-1 and COX-2 selective inhibitors, SC560 and NS398, significantly attenuated constriction at middle and maximal VP concentrations. However, compared with the control RSE group, maximal constriction was reduced to a greater extent by NS398 than by SC560 (65% vs. 36%) indicating a greater role for COX-2 constrictor prostanoids in RSE.

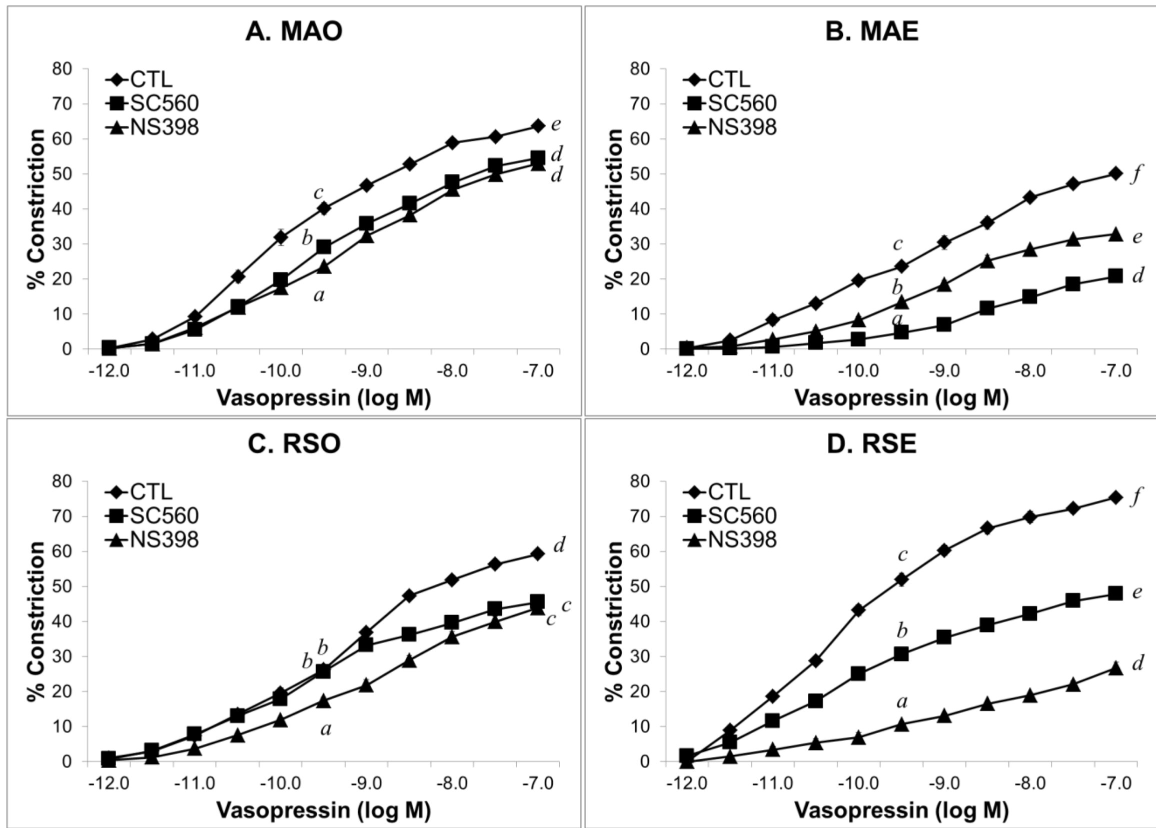

Figure 18. Concentration-response curves for VP in endothelium-intact pressurized middle cerebral artery segments prepared from female Sprague-Dawley rats in the presence of selective COX inhibitors SC560 (COX-1), NS398 (COX-2), or vehicle control.
Vessels were prepared in triplicate from each animal group: mature multigravid adult female rats (MA, 4-6 mo.), either ovariectomized (MAO) **(A)** or ovariectomized and estrogen-replaced (MAE) **(B)**, and reproductively senescent female rats (RS, 10-12 mo.), either ovariectomized (RSO) **(C)** or ovariectomized and estrogen-replaced (RSE) **(D)**. Data points represent means ± SE (n=6-7 rats/group).$^{a\text{-}f}P \leq 0.0001$, mean values without common superscript differ significantly at middle and maximal concentrations of VP.

Table 8. Middle- and Maximal-VP concentration and EC_{50} in MAO, MAE, RSO and RSE.

Group (n=6-7)	Middle VP concentration ($10^{-9.5}$ M, % constriction)	Maximal VP concentration (10^{-7} M, % constriction)	EC_{50} (nM)
Control			
MAO	40.2 ± 1.8^{b}	63.6 ± 0.7^{b}	0.14 ± 0.04^{b}
MAE	23.6 ± 1.3^{a}	50.1 ± 0.9^{a}	0.40 ± 0.15^{bc}
RSO	26.2 ± 1.4^{a}	59.3 ± 1.1^{b}	0.40 ± 0.04^{c}
RSE	52.0 ± 1.9^{c}	75.4 ± 1.1^{c}	0.07 ± 0.01^{a}
SC560			
MAO	29.0 ± 1.3^{b}	54.5 ± 1.1^{c}	0.37 ± 0.07^{c}
MAE	4.7 ± 0.5^{a}	20.8 ± 0.7^{a}	2.72 ± 0.41^{d}
RSO	25.7 ± 0.8^{b}	45.6 ± 1.0^{b}	0.21 ± 0.02^{b}
RSE	30.7 ± 1.4^{b}	47.9 ± 1.0^{b}	0.13 ± 0.04^{a}
NS398			
MAO	23.6 ± 0.9^{b}	53.0 ± 0.8^{a}	0.46 ± 0.10^{a}
MAE	13.4 ± 1.2^{a}	32.8 ± 0.2^{b}	0.98 ± 0.40^{a}
RSO	17.4 ± 1.1^{a}	43.9 ± 1.3^{b}	1.00 ± 0.26^{a}
RSE	10.7 ± 0.9^{ab}	26.8 ± 1.7^{a}	1.02 ± 0.42^{a}

Mature multigravid adult female rats (MA, 4-6 mo.), either ovariectomized (MAO) or ovariectomized and estrogen-replaced (MAE), and reproductively senescent female rats (RS, 10-12 mo.), either ovariectomized (RSO) or ovariectomized and estrogen-replaced (RSE). Values are means ± SE; n=no of animals studied. $^{a\text{-}c}0.0001 \leq P \leq 0.009$, values within columns (Middle VP concentration, Maximal VP concentration, EC_{50}) with different superscripts are significantly different.

5.3.3. Effects of age and sex on basal and VP-stimulated PGI_2 production

Basal and vasopressin-stimulated (low concentration 10^{-9} M; or high concentration 10^{-7} M) release of 6-keto-$PGF_{1\alpha}$ (stable metabolite of PGI_2) are shown in Figure 19 and Table 9. Basal release of 6-keto-$PGF_{1\alpha}$ did not differ significantly among the experimental groups (MAO, MAE, RSO, or RSE). Within all groups VP increased 6-keto-$PGF_{1\alpha}$

production in a concentration-dependent manner. Low VP concentration increased 6-keto-$PGF_{1\alpha}$ production 3 fold in all groups. Age had no significant effect on low concentration VP-stimulation of 6-keto-$PGF_{1\alpha}$ production in ovariectomized or estrogen replaced females (MAO vs. RSO and MAE vs. RSE); however, 6-keto-$PGF_{1\alpha}$ production was significantly increased with estrogen in both ages (MAO vs. MAE and RSO vs. RSE). With advancing age high concentration VP-stimulated production of 6-keto-$PGF_{1\alpha}$ was significantly decreased in RSE as compared to MAE, with no differences in RSO vs. MAO. In contrast, high concentration VP-stimulated 6-keto-$PGF_{1\alpha}$ release was significantly increased with estrogen in both MA and RS (MAO vs. MAE and RSO vs. RSE). High-VP concentration increased 6-keto-$PGF_{1\alpha}$ production 6 fold in MAE and 5 fold in MAO, RSO and RSE from their respective basal levels. At high VP concentration MAE produced significantly more 6-keto-$PGF_{1\alpha}$ than all other groups.

Table 9. Basal, low- and high-VP stimulated PGI_2 in MAO, MAE, RSO and RSE.

Group	Basal (pg/mg/45 min)	Low VP ($10^{-9)}$ (pg/mg/45 min)	High VP (10^{-7}) (pg/mg/45 min)
MAO	4,607 ± 427.4[a]	12,304 ± 2,136[a]	22,805 ± 3,108[a]
MAE	5,646 ± 786.9[a]	18,712 ± 1,913[b]	35,975 ± 2,133[c]
RSO	3,878 ± 744.2[a]	12,818 ± 1,603[a]	18,499 ± 1,563[a]
RSE	5,867 ± 998.0[a]	18,546 ± 1,183[b]	28,796 ± 2,395[b]

Mature multigravid adult female rats (MA, 4-6 mo.), either ovariectomized (MAO) or ovariectomized and estrogen-replaced (MAE), and reproductively senescent female rats (RS, 10-12 mo.), either ovariectomized (RSO) or ovariectomized and estrogen-replaced (RSE). Values are means ± SE; n=6 rats/group. [a-c]$0.0001 \leq P \leq 0.02$, values within columns (Basal, low VP, high VP) with different superscripts are significantly different.

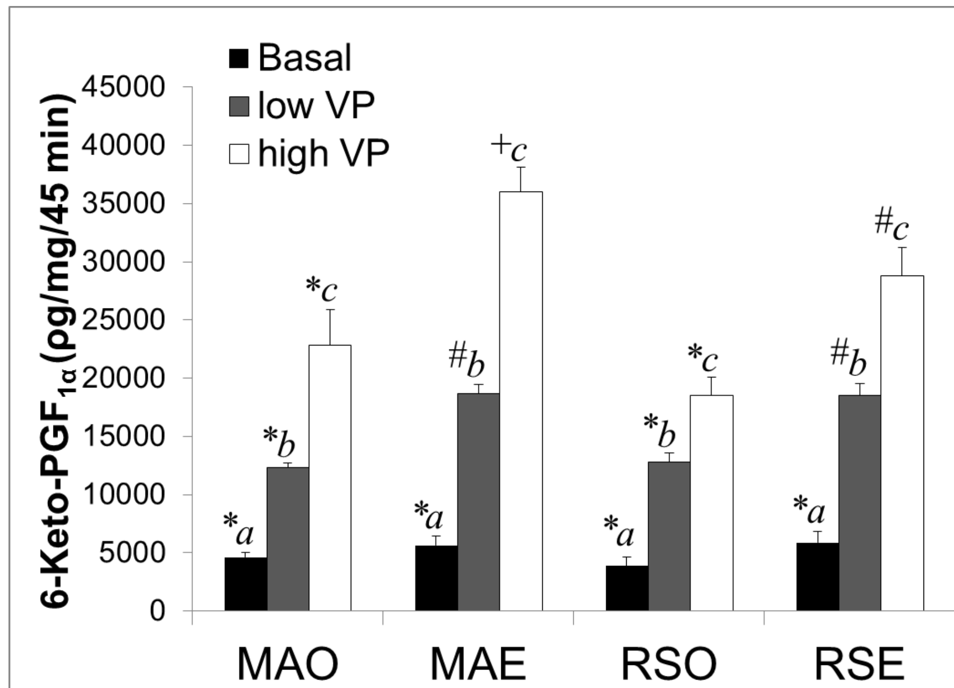

Figure 19. Basal and vasopressin-stimulated (low concentration, 10^{-9} M; or high concentration 10^{-7} M) release of 6-keto-$PGF_{1\alpha}$ by middle cerebral artery segments from MAO, MAE, RSO and RSE rats.

Mature multigravid adult female rats (MA, 4-6 mo.), either ovariectomized (MAO) or ovariectomized and estrogen-replaced (MAE), and reproductively senescent female rats (RS, 10-12 mo.), either ovariectomized (RSO) or ovariectomized and estrogen-replaced (RSE). Values are means ± SE; n=6 rats/group. $^{a\text{-}c}P \leq 0.0001$, mean values within groups (MAO, MAE, RSO, RSE) without common superscript are significantly different. $^{*,\#,+}$ $0.0001 \leq P \leq 0.02$ mean values between groups (MAO vs. MAE vs. RSO vs. RSE) with different superscripts are significantly different.

5.3.4. Effects of age and sex on basal and VP-stimulated TXA_2 production

Basal and vasopressin-stimulated (low concentration 10^{-9} M; high concentration 10^{-7} M) release of TXB_2 (stable metabolite of TXA_2) are shown in Figure 20 and Table 10. Basal release of TXB_2 did not significantly differ between groups (MAO, MAE, RSO, or RSE). In MAO and RSO there was no difference between basal and low concentration-VP TXB_2 production; however, in MAE, estrogen replacement increased TXB_2 production 2 fold in MAE and 3 fold in RSE. Thus, TXB_2 production increased in a

concentration-dependent manner, indicating increased sensitivity to VP with estrogen treatment. Age had no significant effect on low-concentration VP-stimulated TXB_2 release in ovariectomized or estrogen-treated groups (MAO vs. RSO and MAE vs. RSE); however, TXB_2 release was significantly increased with estrogen in both younger and older females (MAO vs. MAE and RSO vs. RSE). TXB_2 production was not different between MAO and RSO; however age and estrogen increased TXB_2 production in older RSE as compared to MAE. High-concentration VP-stimulated TXB_2 release was significantly increased with estrogen in both MA and RS (MAO vs. MAE and RSO vs. RSE). High-VP concentration increased TXB_2 production 3 fold in MAO, 4 fold in MAE, 3 fold in RSO and 7 fold in RSE from their respective basal levels. At high VP concentration RSE produced significantly more TXB_2 than all other groups.

Table 10. Basal, low- and high-VP stimulated TXA_2 in MAO, MAE, RSO and RSE.

Group (n=7-8)	Basal (pg/mg/45 min)	Low VP (10^{-9}) (pg/mg/45 min)	High VP (10^{-7}) (pg/mg/45 min)
MAO	242.8 ± 30.4[a]	325.4 ± 46.2[a]	670.9 ± 57.4[a]
MAE	302.8 ± 75.5[a]	618.6 ± 76.6[b]	1,085 ± 70.0[b]
RSO	202.6 ± 37.6[a]	262.8 ± 70.4[a]	527.1 ± 71.1[a]
RSE	187.1 ± 37.6[a]	468.0 ± 78.7[b]	1,397 ± 73.2[c]

Mature multigravid adult female rats (MA, 4-6 mo.), either ovariectomized (MAO) or ovariectomized and estrogen-replaced (MAE), and reproductively senescent female rats (RS, 10-12 mo.), either ovariectomized (RSO) or ovariectomized and estrogen-replaced (RSE). Values are means ± SE; n=6 rats/group. [a-c]$0.0001 \leq P \leq 0.003$, values within columns (Basal, low VP, high VP) with different superscripts are significantly different.

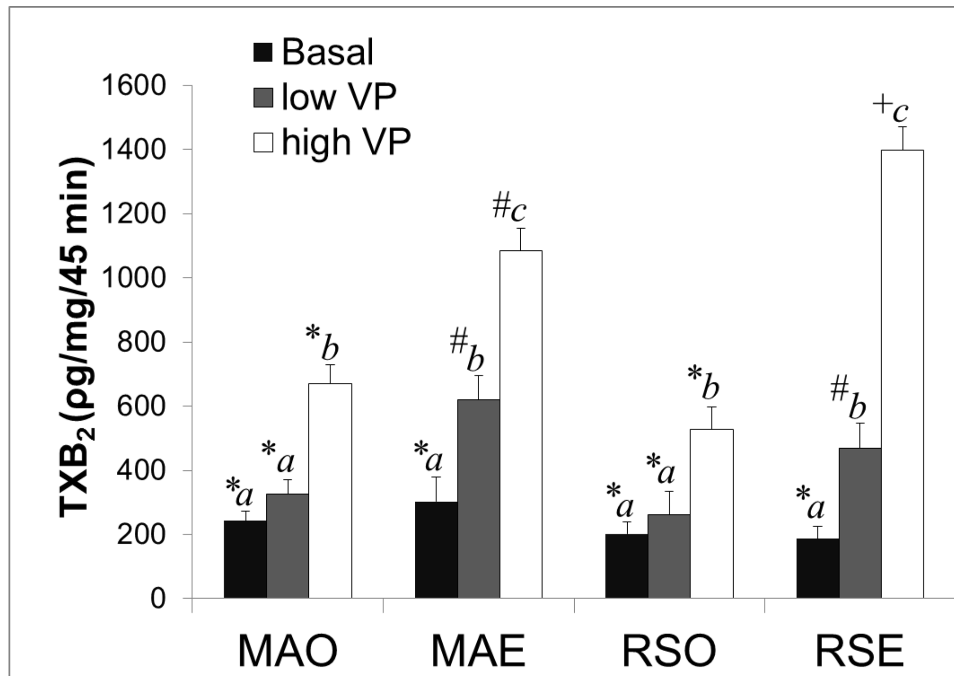

Figure 20. Basal and vasopressin-stimulated (low concentration, 10^{-9} M; or high concentration 10^{-7} M) release of TXB_2 by middle cerebral artery segments from MAO, MAE, RSO and RSE rats. Mature multigravid adult female rats (MA, 4-6 mo.), either ovariectomized (MAO) or ovariectomized and estrogen-replaced (MAE), and reproductively senescent female rats (RS, 10-12 mo.), either ovariectomized (RSO) or ovariectomized and estrogen-replaced (RSE). Values are means ± SE; n=6 rats/group. $^{a\text{-}c}0.0001 \leq P \leq 0.003$, mean values within groups (MAO, MAE, RSO, RSE) without common superscript are significantly different. $^{*,\#,+}$ $0.0001 \leq P \leq 0.003$ mean values between groups (MAO vs. MAE vs. RSO vs. RSE) with different superscripts are significantly different.

5.4. Discussion

In the present investigation, the effects of age and exogenous estrogen on cerebrovascular reactivity and prostanoid production were examined in the MCA of ovariectomized and estrogen-replaced female Sprague-Dawley rats. The results reveal that estrogen is an important regulator of the vasoconstrictor responses of the MCA to VP in the female rat and that it exerts opposing effects on cerebrovascular reactivity with advancing age. The main findings of this study are that: 1) estrogen treatment had

divergent effects on VP-induced cerebrovascular vasoconstriction, 2) age altered the specific COX isoform and resultant prostanoids enhanced by estrogen, and 3) estrogen enhanced VP-stimulated PGI_2 and TXA_2 production in both MA and RS, while age decreased VP-stimulated PGI_2 and increased in TXA_2 production in females with estrogen replacement. Taken together, these data reveal that the vascular effects of estrogen are distinctly age-dependent. In younger ovariectomized MA females with estrogen replacement, the beneficial and protective effects of estrogen are very evident (decreased vasoconstriction, increased dilator function). Conversely, in older ovariectomized RS females with estrogen replacement, the detrimental effects of estrogen begin to be manifested (enhanced vasoconstriction, increased constrictor prostanoid function). These findings contribute to the understanding of the interactive effects of age and estrogen on the modulation of cerebrovascular function.

To determine the roles of age and estrogen on the regulation of VP- induced vasoconstriction of female rat middle cerebral artery, the effects of ovariectomy and estrogen replacement therapy on vascular reactivity to VP and prostanoid function were determined. Plasma concentrations of 17β-estradiol were significantly attenuated by ovariectomy in both age groups. Estrogen replacement therapy restored plasma estradiol levels to concentrations that did not differ between MA or RS groups and are consistent with estradiol levels in intact female rats previously reported (Li *et al.*, 2005; Ospina *et al.*, 2002).

5.4.1. Age-dependent effects on the modulation of cerebrovascular reactivity

Endothelial dysfunction increases in men after age 40 and in women after age 55 (Celermajer *et al.*, 1994). While the exact cause of the decline in endothelial function is unknown, aging is usually associated with a reduction in the ability to elicit endothelium-dependent vasodilation in both animals and in humans (Csiszar *et al.*, 2002; Taddei *et al.*, 2001; Tschudi *et al.*, 1996). This loss of function occurs via numerous mechanisms related to nitric oxide-mediated dilation including: increased activity of arginase, augmented production of oxygen derived free radicals, reduced expression of eNOS, lesser eNOS activity, reduced expression of soluble guanylyl cyclase, and decreased nitric oxide release (Csiszar *et al.*, 2002; Kloss *et al.*, 2000; Taddei *et al.*, 2001; Tschudi *et al.*, 1996). In addition to the age-related changes in nitric oxide, an enhancement of vasoconstrictor prostaglandins further potentiates age-dependent endothelial dysfunction. Both COX-1 and COX-2 expression are upregulated with advancing age (Heymes *et al.*, 2000; Kang *et al.*, 2007; Matz *et al.*, 2000; Numaguchi *et al.*, 1999; Stewart *et al.*, 2000; Tang *et al.*, 2008). In contrast, with advancing age PGI_2 receptor (IP) expression declines, reducing the efficacy of PGI_2 that is produced (Ge *et al.*, 1995; Numaguchi *et al.*, 1999; Shi *et al.*, 2008; Tang *et al.*, 2008). There is also indirect evidence suggesting that untransformed PGH_2 is augmented with aging due to COX-1/COX-2 upregulation (Dai *et al.*, 1992; Hynes *et al.*, 1987; Lin *et al.*, 1994; Pagano *et al.*, 1991). Numerous studies have reported increases in TXA_2 and TXS mRNA in aorta and mesenteric arteries with age (Matz *et al.*, 2000; Tang *et al.*, 2008).

Interestingly, in the current study, advancing age had no effect on VP-induced vasoconstriction in ovariectomized rats; however, in estrogen replaced rats, advancing age significantly augmented VP-induced vasoconstriction. Advancing age also altered the specific COX isoform and resultant prostanoids enhanced by estrogen; COX-1 in younger MA females, COX-2 in older RS females with estrogen replacement. Furthermore, advancing age decreased VP-stimulated PGI_2 production and increased VP-stimulated TXA_2 production with estrogen replacement. Thus, in agreement with previous studies, the balance of dilator to constrictor prostanoids appears to be altered with age, including decreased release and/or reactivity to PGI_2 and augmentation of constrictor prostanoid PGH_2 and TXA_2 production.

5.4.2. Effect of estrogen on mechanisms of cerebrovascular reactivity

Many studies have reported that estrogen enhances the production and/or the sensitivity of cerebral arteries to vasodilatory factors nitric oxide and PGI_2 (Geary *et al.*, 2000a; Geary *et al.*, 1998; Ospina *et al.*, 2003; Ospina *et al.*, 2002; Pelligrino *et al.*, 2000; Skarsgard *et al.*, 1997). In the cerebrovasculature, estrogen appears to shift the prostaglandin balance towards greater production of vasodilator prostanoids (Ospina *et al.*, 2003). In cerebral vessels of young rats, estrogen elevates both COX-1 and PGIS resulting in enhanced PGI_2 production (Geary *et al.*, 2000a; Ospina *et al.*, 2003; Ospina *et al.*, 2002). Interestingly TXA_2 production was also shown to be slightly yet significantly elevated in young animals with estrogen-treatment, perhaps due to increased COX-1 levels (Lin *et al.*, 2002; Ospina *et al.*, 2002).

The current study reveals that estrogen is an important regulator of VP-induced vasoconstriction of the female rat middle cerebral artery and that the effects of estrogen on cerebrovascular reactivity appear to differ with advancing age. In younger MA females, estrogen markedly attenuated vasoconstrictor responses to VP. In contrast, in older RS females, estrogen significantly augmented cerebrovascular vasoconstrictor responses to VP. Thus, estrogen exerts divergent effects on the modulation of cerebrovascular function with advancing age. The second major finding was that estrogen enhanced the role of COX-1 and COX-2 prostanoid pathways in VP-induced cerebrovascular vasoconstriction. In younger MA females with estrogen replacement, SC560 reduced reactivity to a greater extent than NS398, indicating that COX-1 derived prostanoids were dominant. In contrast, in older RS females receiving estrogen replacement this effect was reversed with NS398 attenuating contraction by a greater extent than SC560, suggesting a dominant role for COX-2 derived prostanoids with advancing age. Furthermore, VP-stimulated PGI_2 and TXA_2 production were enhanced significantly by estrogen replacement in both younger and older ovariectomized females. Taken together with results from the functional data, the attenuation of VP-induced vasoconstriction in younger MA females appears to be due to estrogen-enhanced production of PGI_2, while the age-dependent augmentation of vasoconstriction in older females is due to enhanced production of TXA_2. These data provide evidence that estrogen exerts different effects depending on age. In younger females, estrogen replacement therapy is beneficial; yet, in older females, estrogen replacement therapy is detrimental. These findings help to explain why estrogen is beneficial and

neuroprotective in young animals but deleterious in postmenopausal women. The age- and estrogen-dependent shifts in dilator and constrictor prostanoids observed in the present study reveal important novel roles for estrogen and prostanoids in cerebrovascular function during aging not previously reported.

5.4.3. Beneficial and deleterious effects of estrogen and age on cerebrovascular function

Earlier findings of human epidemiological (Kawas *et al.*, 1997; Levy *et al.*, 1988; Messerli *et al.*, 1987) and experimental animal studies (Farhat *et al.*, 1996; Karas, 2002; Simpkins *et al.*, 1997) led to the belief that estrogen replacement therapy exerts beneficial effects on neurological and cardiovascular health and is protective against diseases such as dementia, coronary artery disease, hypertension, and stroke. An abundance of evidence from experimental animal studies has established that estrogen does exert beneficial or protective effects on the cerebrovasculature by reducing vascular reactivity and thereby increasing blood flow through nitric oxide- and vasodilator prostanoid-dependent mechanisms (Geary *et al.*, 2000a; Geary *et al.*, 1998; McNeill *et al.*, 1999; McNeill *et al.*, 2002; Orshal *et al.*, 2004; Osanai *et al.*, 2000; Ospina *et al.*, 2003; Ospina *et al.*, 2002). In contrast, more recent human epidemiological findings such as the HERS (Hulley *et al.*, 1998), HERSII (Grady *et al.*, 2000; Hulley *et al.*, 2002), and WHI studies (Hendrix *et al.*, 2006; Wassertheil-Smoller *et al.*, 2003) all suggest that in older women estrogen replacement therapy increases the incidences of neurological (dementia and stroke) and vascular diseases (coronary artery disease,

hypertension, and venous thrombosis). Thus, the role of estrogen in cardiovascular health and disease has become controversial.

Numerous studies have revealed age-related changes in vascular function due to increases in oxidative stress. Advancing age impairs eNOS-dependent vasodilation by increases in superoxide formation via activation of NADPH oxidase in pial arterioles from male rats (Mayhan *et al.*, 2008). Similar studies in the cerebral vasculature of young and aged female rats showed that estrogen replacement therapy significantly reduced bacterial lipopolysaccharide induced increases in iNOS and COX-2 formation in young but not in older animals (Sunday *et al.*, 2007). Since both iNOS and COX-2 can lead to the formation of ROS, vasoconstrictor tone of cerebral vessels would thus be enhanced in aged as compared to younger female rats. Age-dependent decreases in the coupled state of NOS may also occur. In the uncoupled state, NOS catalyzes NADPH to superoxide ion, rather than producing nitric oxide. Additionally, there is evidence that aging reduces the levels of L-arginine (Berkowitz *et al.*, 2003) and tetrahydrobiopterin (Carlstrom *et al.*, 2009) providing a greater potential for uncoupling of NOS and increased production of superoxide (White *et al.*, 2005). These findings provide a clear mechanistic explanation for an age-dependent change in the effects of estrogen on the vascular wall, from the beneficial expression of eNOS and formation of nitric oxide in younger animals to the uncoupling of eNOS and the deleterious formation of superoxide in older animals. In summary, in younger females, estrogen leads to the formation of beneficial or protective substances such as nitric oxide; however, with advancing age

estrogen therapy may result in the formation of detrimental substances including increased formation of superoxide and other ROS.

Although multiple studies have demonstrated beneficial effects of estrogen replacement in numerous vascular beds of young animals (Geary *et al.*, 2000a; Li *et al.*, 2008; Ospina *et al.*, 2003), this is the first study to compare the effects of estrogen replacement in female rats of younger and more advanced age. Further understanding of the mechanisms underlying the effects of age and estrogen on the regulation of cerebrovascular function will aid in the knowledge of their contributions in pathophysiological states such as stroke.

6. SUMMARY AND CONCLUSIONS

The purpose of this dissertation was to investigate the role of two therapeutic interventions (exercise training and hormone replacement therapy) on differing states of endothelial dysfunction (chronic coronary occlusion and aging). The first project utilized a model of chronic coronary artery occlusion to evaluate the effects of exercise training on cellular and molecular adaptations of the nonoccluded control and collateral-dependent coronary vasculature. The second project utilized a model of aging to evaluate the interactive effects of age and hormone replacement therapy on the cellular mechanisms underlying the regulation of cerebrovascular function.

6.1. Normal endothelial function

Under normal conditions, the vascular endothelium regulates of a number of important vascular functions, including thrombosis, smooth muscle cell proliferation, vascular tone, cell migration, leukocyte adhesion, and inflammatory responses, by producing numerous endothelium-derived vasoactive mediators (Feletou *et al.*, 2006; Landmesser *et al.*, 2004; Vane *et al.*, 1990; Vanhoutte *et al.*, 2009). In response to mechanical or humoral stimuli, the endothelium releases several agents that regulate vascular function, including: nitric oxide, PGI_2, EDHFs, TXA_2, and ROS. The release of nitric oxide and other vascular mediators by endothelial cells can be modulated by both acute and chronic factors. Enhanced secretion or up-regulation of vascular mediators occurs via increased shear stress, hormones, exercise, and diet, while decreased secretion or down-

regulation occurs via oxidative stress, smoking, obesity, and vascular diseases (Vanhoutte *et al.*, 2009).

6.2. Endothelial dysfunction

Endothelial dysfunction is exhibited in many disease states including coronary artery disease, hypertension, and diabetes, and is also associated with advancing age. It is characterized by a decrease in nitric oxide bioavailability (decreased synthesis or increased degradation) and an increase in the production of constrictor prostanoids. Endothelial dysfunction alters vascular health in multiple ways including decreased nitric oxide bioactivity, increased superoxide production, attenuation of endothelium-dependent dilation, increased vascular tone, and atherogenesis (Feletou *et al.*, 2006; Lyons, 1997; Vanhoutte, 1997; Vanhoutte, 1998; Vanhoutte *et al.*, 2009). The loss of nitric oxide bioavailability is a key manifestation of endothelial dysfunction that contributes to the pathogenesis and clinical expression of many disease states. Its presence predicts the severity of outcome, particularly the occurrence of myocardial infarction and stroke (Suwaidi *et al.*, 2000; Vanhoutte, 1997).

6.2.1. Chronic coronary occlusion

Endothelial dysfunction is a key characteristic of cardiovascular disease. Its presence predicts the severity of outcome, particularly the occurrence of myocardial infarction (Suwaidi *et al.*, 2000; Vanhoutte, 1997). In patients with coronary artery disease, treatment with aspirin (COX inhibitor) and a TP receptor antagonist results in improved

endothelial function (Belhassen *et al.*, 2003; Husain *et al.*, 1998). These findings suggest that endothelium-derived prostanoids contribute to endothelial dysfunction in patients with coronary artery disease. Additionally, decreased synthesis and/or increased degradation leads to a decrease in the bioavailability of nitric oxide, which is a key manifestation of endothelial dysfunction that contributes to the pathogenesis of atherosclerosis (Vita, 2011).

The effects of chronic coronary occlusion on coronary vascular function were examined in the first project of this dissertation. Interestingly there were no significant differences between the collateral-dependent LCX and nonoccluded control LAD of sedentary animals. This suggests that collateral-development was sufficient enough to provide adequate flow into the compromised LCX artery to maintain normal function.

6.2.2. Aging

Endothelial dysfunction increases in men after age 40 and in women after age 55 (Celermajer *et al.*, 1994). While the exact causes of age-dependent decreases in endothelial function are unknown, aging is usually associated with a reduction in the ability of the endothelium to elicit endothelium-dependent vasodilation in both animals and humans (Csiszar *et al.*, 2002; Taddei *et al.*, 2001; Tschudi *et al.*, 1996). This loss of function occurs via numerous mechanisms related to nitric oxide-mediated dilation including: increased activity of arginase, augmented production of oxygen derived free radicals, reduced expression of eNOS, lower eNOS activity, reduced expression of

soluble guanylyl cyclase, and decreased nitric oxide release (Csiszar *et al.*, 2002; Kloss *et al.*, 2000; Taddei *et al.*, 2001; Tschudi *et al.*, 1996). Numerous studies have revealed age-related changes in vascular function due to increases in oxidative stress. Advancing age impairs eNOS-dependent vasodilation by increases in superoxide formation via activation of NAD(P)H oxidase in pial arterioles from male rats (Mayhan *et al.*, 2008). Additionally, estrogen replacement therapy significantly reduced bacterial lipopolysaccharide induced increases in iNOS and COX-2 formation in young but not in older animals (Sunday *et al.*, 2007), leading to increased formation of ROS, and enhanced vasoconstrictor tone of cerebral vessels with advancing age. Aging reduces the levels of L-arginine (Berkowitz *et al.*, 2003) and tetrahydrobiopterin (Carlstrom *et al.*, 2009) providing a greater potential for uncoupling of NOS and increased production of superoxide (White *et al.*, 2005) rather than nitric oxide. These findings provide a clear mechanistic explanation for an age-dependent change in the effects of estrogen on the vascular wall, from the beneficial expression of eNOS and formation of nitric oxide in younger animals to the uncoupling of eNOS and the deleterious formation of superoxide in older animals.

In addition, an enhancement of vasoconstrictor prostanoids potentiates age-dependent endothelial dysfunction. With advancing age, both COX-1 and COX-2 expression are upregulated by oxidative stress. Furthermore, PGI_2 receptor (IP) expression decreases with age (Ge *et al.*, 1995; Numaguchi *et al.*, 1999; Shi *et al.*, 2008; Tang *et al.*, 2008). There is indirect evidence suggesting that untransformed PGH_2 is also augmented with

aging due to COX-1 and/or COX-2 upregulation (Dai *et al.*, 1992; Hynes *et al.*, 1987; Lin *et al.*, 1994; Pagano *et al.*, 1991). Numerous studies have shown increases in TXA_2 and TXS mRNA in aorta and mesenteric arteries with age (Matz *et al.*, 2000; Tang *et al.*, 2008). Thus, the balance of dilator to constrictor prostanoids is altered with age, involving decreased sensitivity to PGI_2 and enhanced production of constrictor prostanoids PGH_2 and TXA_2, which eventually leads to endothelial dysfunction.

The effects of advancing age on cerebrovascular function were examined in the second project of this dissertation. First, the effects of age and sex on VP-induced vasoconstriction were examined using intact male and female Sprague-Dawley rats of two age groups. Interestingly, VP-induced vasoconstriction was enhanced with age in intact female but was unchanged in male rats. In intact rats of both sexes, selective blockade of COX-1 or COX-2 pathways produced age-dependent changes in cerebrovascular reactivity to VP. Additionally, VP-stimulated PGI_2 and TXA_2 production significantly decreased with age in intact females but was unchanged in males. Second, the mechanisms underlying the effects of age and estrogen on VP-induced vasoconstriction were examined using ovariectomized and estrogen-replaced female Sprague-Dawley rats of two age groups. Age had no effect on VP-induced vasoconstriction in ovariectomized rats; however, in estrogen replaced rats, advancing age significantly augmented VP-mediated vasoconstriction. Advancing age also altered the specific COX isoform and resultant prostanoids enhanced by estrogen; COX-1 in younger MA females, COX-2 in older RS females with estrogen replacement.

Furthermore, advancing age decreased VP-stimulated PGI_2 production and increased VP-stimulated TXA_2 production in female rats with estrogen replacement.

6.3. Effects of therapeutic interventions on endothelial function

Numerous therapeutic interventions, including antioxidants, lipid-lowering drugs, exercise, and hormone replacement therapy, are known to improve coronary and peripheral endothelial function.

6.3.1 Exercise training

The effect of exercise on the vascular health of patients with coronary artery disease is of considerable interest and the benefits of regular exercise following a cardiac event are quite significant. Exercise-based cardiac rehabilitation for patients with CAD decreases total mortality by 20% and cardiac mortality by 26% (Taylor *et al.*, 2004). Participation in a comprehensive cardiac exercise-based rehabilitation program leads to a significant reduction in cardiac events and hospital readmissions and a significant increase in functional capacity (Ades *et al.*, 1997; Hedback *et al.*, 2001).

The first project of this dissertation examined the therapeutic intervention of exercise training. The results from these studies revealed the novel finding that exercise training enhances multiple mechanisms of endothelium-dependent vascular relaxation which appear to function in a compensatory manner rather than in an additive fashion. Bradykinin-stimulated nitric oxide levels were significantly increased after exercise

training in endothelial cells of both nonoccluded and collateral-dependent arteries. Bradykinin treatment significantly increased PGI_2 levels in all artery treatment groups and PGI_2 production tended to be further enhanced after NOS inhibition in exercise-trained pigs. There was no effect of chronic occlusion or exercise training on K^+ channel or BK_{Ca} channel currents. Furthermore, there was no effect of occlusion or exercise training on BK_{Ca} channel protein levels in these studies. In contrast, the effect of IBTX in the functional data suggests that BK_{Ca} channels do contribute to the enhanced relaxation after exercise. Taken together, these data suggest that a bradykinin-sensitive cellular signaling pathway that mediates smooth muscle relaxation via BK_{Ca} channels may be upregulated by exercise training and contribute to enhanced relaxation, rather than an increase in BK_{Ca} channel protein. This pathway may be a putative EDHF since the contribution of BK_{Ca} channels persisted in the presence of NOS and prostanoid inhibition. Interestingly, despite increases in these signaling molecules with exercise training, bradykinin-mediated, endothelium-dependent relaxation was not enhanced in arteries from exercise-trained pigs. Bradykinin-mediated relaxation was, however, more persistent in arteries from exercise-trained pigs after inhibition of select endothelium-dependent signaling molecules, suggesting redundancy in signaling pathways of vascular relaxation. These data provide evidence that multiple vasodilators contribute to exercise training-enhanced endothelium-dependent relaxation in coronary arteries of hearts exposed to chronic occlusion. Although redundant, upregulation of multiple parallel regulatory systems may provide more refined control of blood flow after exercise training.

6.3.2. Hormone replacement therapy

Since the late 1900s, estrogen has been known to exert neuroprotective effects in animal models of cerebral ischemia or stroke. Younger premenopausal women are protected from ischemic stroke as compared to males, yet this protective effect is lost after menopause. Additionally, premenopausal women experience less damage and greater functional and cognitive recovery from neurologic insult than males. Because of these findings, as well as supporting evidence from animal studies, exposure to estrogen was postulated to be neuroprotective. In 2000, an estimated 10 million women were receiving HRT for the alleviation of menopausal symptoms. However, after just a few years, reports from WHI indicated that estrogen therapy significantly increased incidence and severity of stroke. Thus, it is important to examine why estrogen is beneficial and neuroprotective in young animals but not in postmenopausal women.

The second project of this dissertation examined the therapeutic intervention of hormone replacement therapy. The results from these studies revealed the novel finding that estrogen is an important regulator of the constrictor responses of the female rat middle cerebral artery to VP and that its effects on cerebrovascular reactivity appear to differ with advancing age. In younger MA females, estrogen markedly attenuated vasoconstrictor responses to VP. In contrast, in older RS females, estrogen significantly augmented cerebrovascular vasoconstrictor responses to VP. Thus, estrogen exerts divergent effects on the modulation of cerebrovascular function with advancing age. The second major finding was that estrogen enhanced the role of COX-1 and COX-2

mediated prostanoid pathways in VP-induced cerebrovascular vasoconstriction. In younger MA females with estrogen replacement, SC560 reduced reactivity to a greater extent than NS398, indicating that COX-1 prostanoids were dominant. In contrast, in older RS females receiving estrogen replacement this effect was reversed with NS398 attenuating contraction by a greater extent than SC560, suggesting a dominant role for COX-2 prostanoids with advancing age. Furthermore, VP-stimulated PGI_2 and TXA_2 production were enhanced significantly by estrogen replacement in both younger and older ovariectomized females. Taken together with results from the functional data, the attenuation of VP-induced vasoconstriction in younger MA females appears to be due to estrogen-enhanced production of PGI_2, while the age-dependent augmentation of vasoconstriction in older females is due to enhanced production of TXA_2. These data provide evidence that estrogen exerts different effects depending on age. In younger females, estrogen replacement therapy is beneficial; yet, in older females, estrogen replacement therapy is detrimental. These findings begin to explain why estrogen is beneficial and neuroprotective in young animals but not in postmenopausal women.

6.4. Clinical significance

Despite remarkable evidence for the therapeutic benefits of physical activity, the mechanisms by which regular exercise improves vascular function in the setting of coronary artery disease are not fully understood. The findings from this study provide new evidence that exercise training concomitantly enhances the contribution of multiple vasodilating mechanisms, including nitric oxide, prostacyclin and BK_{Ca} channels, to

vascular function in ischemic heart disease. Increased contribution of multiple vasodilator signaling pathways after exercise training appears to promote compensation or redundancy to ensure adequate vasodilation and maintenance of coronary blood flow. The results from this project further understanding of the mechanisms underlying coronary vascular adaptations to exercise in health and coronary artery disease.

Similarly, the mechanisms underlying the beneficial effects of estrogen on cerebrovascular function have been studied at length, while the mechanisms responsible for age-dependent deleterious effects of estrogen are largely unknown. Furthermore, the effects of advancing age and estrogen replacement on the modulation of cerebrovascular function are poorly understood. This lack of understanding emphasizes the importance of examining the cellular and molecular mechanisms underlying the role of age in the deleterious effects of estrogen replacement therapy and endogenous estrogen on the cerebral vasculature. The results of this study reveal important and novel information regarding the effects of estrogen and advancing age on cerebrovascular function. Further understanding of the mechanisms by which estrogen exerts beneficial versus detrimental effects on the cerebrovasculature will perhaps lead to new gender-specific therapeutic agents designed specifically to target the cerebrovascular system and other estrogen-responsive tissues.

In summary, the goal of this dissertation research was to investigate the role of two therapeutic interventions (exercise training and hormone replacement therapy) on two

states of endothelial dysfunction (chronic coronary occlusion and aging). The progression from a healthy functional endothelium to a dysfunctional endothelium underlies the development of numerous cardiovascular diseases. In humans, all major cardiovascular risk factors, including hypercholesterolemia, hypertension, diabetes, and smoking, have been associated with endothelial dysfunction. The use of therapeutic interventions to restore endothelial function to a healthy state is a major area of clinical interest. A further understanding of the mechanisms underlying the beneficial effects of these therapeutic interventions will aid in developing better clinical treatments of endothelial dysfunction which underlie many states of disease.

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