NADPH Oxidase as a Mechanistic Link Between Erectile Dysfunction, Peripheral, and Coronary Endothelial Dysfunction in Obesity

by

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Cardiovascular complications involving both microvascular and macrovascular tissues are the major cause of morbidity and mortality in obese patients. Clinical and epidemiological studies suggest that erectile dysfunction and peripheral endothelial dysfunction may predict cardiovascular risk. The purpose of these studies was to investigate NADPH oxidase, a major source of vascular derived oxidative stress, as a common mechanism between erectile dysfunction and coronary artery endothelial dysfunction in rats fed a Western diet (WD), and peripheral endothelial dysfunction in an obese group of human subjects. Male Sprague-Dawley rats were fed a control diet (CD) or WD for 4, 8, or 12 weeks. Erectile function was evaluated by measuring intracavernosal pressure in response to electrical field stimulation of the cavernosal nerve. Coronary artery endothelial function (CAEF) was evaluated *ex vivo* with cumulative doses of (ACh) applied to pre-constricted segments of the left anterior descending coronary artery. Erectile function was significantly attenuated following 8-weeks ($P < 0.05$) and 12-weeks ($P < 0.05$) of the WD, whereas CAEF was significantly attenuated following 12-weeks of the WD ($P < 0.01$). Larger groups of rats were then fed the CD or WD for 12 weeks, and erectile function and CAEF were evaluated following intracavernosal injection or vessel incubation with apocynin, an NADPH oxidase inhibitor. Apocynin improved erectile function ($P < 0.01$) and

CAEF ($P < 0.05$) in WD, and had no effect on CD rats. Sedentary, young adults were recruited across a body mass index (BMI) range of 18-40, and split into BMI tertiles. Microdialysis probes were inserted into the vastus lateralis, and *in vivo* reactive oxygen species (ROS) production was measured, and microvascular endothelial function was assessed via local ACh-stimulated blood flow. ROS production ($P < 0.05$) was elevated and ACh-stimulated blood flow ($P < 0.05$) was attenuated in the highest BMI tertile compared to both other tertiles. Apocynin had a greater effect of attenuating ROS production ($P < 0.05$) and augmenting ACh-stimulated blood flow ($P < 0.05$) in the highest compared to lowest BMI tertile. These studies suggest that NADPH oxidase contributes to obesity associated erectile dysfunction, peripheral endothelial dysfunction, and coronary artery disease development.

NADPH OXIDASE AS A MECHANISTIC LINK BETWEEN ERECTILE DYSFUNCTION, PERIPHERAL, AND CORONARY ENDOTHELIAL DYSFUNCTION IN OBESITY

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TABLE OF CONTENTS

LIST OF TABLES/FIGURES

LIST OF SYMBOLS OR ABBREVIATIONS

Ach	Acetylcholine
Akt	Protein kinase B
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate activated protein kinase
ApoE	Apolipoprotein E
AUC	Area-under-the-curve
BAEC	Bovine aortic endothelial cell
BH_2	Dihydrobiopterin
BH_3^+	Trihydrobiopterin radical
BH_4	Tetrahydrobiopterin
BMI	Body mass index
BSA	Bovine serum albumin
CAD	Coronary artery disease
Ca^{2+}	Calcium ion
cGMP	Cyclic guanosine monophosphate
CVD	Cardiovascular disease
DHFR	Dihydrofolate reductase

dl	Deciliter
DNA	Deoxyribonucleic acid
DOCA	Deoxycorticosterone acetate
DPI	Diphenylene iodonium
ED	Erectile dysfunction
EFS	Electrical field stimulation
ELISA	Enzyme-linked immunosorbant assay
eNOS	Endothelial nitric oxide synthase
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
g	Gram
$gp91^{phox}$	91 kilodalton phagocyte oxidase
GTPase	Guanosine triphosphatase
GTPCH-1	Guanosine triphosphate cyclohydrolase-1
HDL-C	High-density lipoprotein cholesterol
HNE	4-hydroxynonenal
HOCl	Hypochlorous acid

HOO^-	Hydroperoxyl radical
Hsp90	Heat shock protein 90
hum	Human
HUVEC	Human umbilical vein endothelial cell
H_2O_2	Hydrogen peroxide
ICP	Intracavernosal pressure
IIEF	International index of erectile function
kcal	Kilocalorie
kg	Kilogram
KPSS	Potassium supplemented physiological saline solution
l	Liter
LDL-C	Low-density lipoprotein cholesterol
LDLR	Low density lipoprotein receptor
L-NAME	L-NG-Nitroarginine methyl ester
LZR	Lean Zucker rat
m	Meter
M	Molar

MAP	Mean arterial pressure
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
mRNA	Messenger ribonucleic acid
MUFA	Mono-unsaturated fatty acid
NADH	Nicotinamide adenine dinucleotide (reduced)
NADPH	Nicotinamide adenine dinucleotide phosphate
nM	Nanomolar
NMDA	N-methyl D-aspartate
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NO_2^-	Nitrite
$ONOO^-$	Peroxynitrite
OZR	Obese Zucker rat

O_2	Molecular oxygen
O_2^-	Superoxide anion
PBS	Phosphate buffered saline
PE	Polyethylene
PKA	Cyclic AMP-dependent protein kinase
PKC	Protein kinase C
PKG	cGMP dependent protein kinase
PKC-ζ	Protein kinase C-Zeta
pM	Picomolar
PP1	Serine-threonine protein phosphatase I
PSS	Physiological saline solution
PUFA	Poly-unsaturated fatty acid
$p22^{phox}$	22 kilodalton phagocyte oxidase
$p40^{phox}$	40 kilodalton phagocyte oxidase
$p47^{phox}$	47 kilodalton phagocyte oxidase
$p67^{phox}$	67 kilodalton phagocyte oxidase
Rac	Rho-related C3 botulinum toxin substrate

ROS	Reactive oxygen species
Ser	Serine
SFA	Saturated fatty acid
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
STZ	Streptozotocin
TBARS	Thiobarbituric acid reactive substances
TBHQ	Tert-butylhydroquinone
TC	Total-cholesterol
TG	Triglycerides
Thr	Threonine
TMBZ	Tetramethylbenzene
V	Volts
VEGF	Vascular endothelial growth factor
VO_{2max}	Maximal aerobic capacity
WD	Western diet
XO	Xanthine oxidase

µIU	Micro International Unit
µl	Microliter
µM	Micromolar
5-HT	5-hydroxytryptamine

CHAPTER 1: INTRODUCTION

Obesity is a major health care concern in the United States, the prevalence of which has risen from 19% in 1997 to over 33% in 2008 (1). Furthermore, obesity is no longer strictly an American problem. Obesity rates have drastically risen in Western and Central Europe since the 1980's, and continue to rise in much of the developing world (2). Cardiovascular complications involving both microvascular and macrovascular tissues are the major cause of morbidity and mortality in obese patients (3). Obesity has shown to be an independent risk factor for coronary (4) and systemic (5) endothelial dysfunction, which are detectable before the onset of diabetes (6). Endothelial dysfunction is a term that encompasses various functional alterations in the vascular endothelium, such as impaired vasodilation, angiogenesis, barrier dysfunction, and inflammatory activation. All of these functional alterations often occur early in the course of vascular diseases associated with obesity and the metabolic syndrome such as atherosclerosis, hypertension, and diabetes (7, 8). A dysfunctional endothelium induces vasoconstriction, platelet aggregation, monocyte adhesion, thrombosis, and inflammation, all of which promote the formation of vascular lesions (9, 10). Impaired vasodilator responses at the level of the resistance vessels leading to the nutritive capillary beds develop progressively, together with an increase in adiposity (11-13). Thus, obesity may promote the development of endothelial dysfunction throughout the vascular tree. With very limited access to the human coronary vasculature, understanding developmental patterns of endothelial dysfunction in various vascular beds in response to obesity is potentially powerful with respect to CVD risk assessment and prevention. Furthermore, development of innovative, minimally invasive approaches to studying the peripheral vasculature will aid in understanding of mechanistic aspects of vascular disease development.

Epidemiological studies indicate that obesity is a significant, independent risk factor for erectile dysfunction (ED) (14-16). Reduction in erectile function may be an early indication of cardiovascular risk as patients with ED are twice as likely to develop major adverse cardiac events compared to those without ED (17). Recognition of reduced erectile function may be an important indicator of future adverse cardiac events, as clinical trial data suggests that the presence of ED in otherwise healthy men may be associated with early, subclinical signs of coronary artery disease (CAD) that may not be detectable during stress testing (18-21). The time interval between the onset of ED symptoms and the occurrence of CAD symptoms is estimated at 2-3 years, and an estimated 3-5 years between onset of ED and a cardiovascular event (myocardial infarction or stroke) (22-24). Additionally, multiple regression analysis indicates that ED is the most efficient predictor of silent CAD in apparently uncomplicated type 2 diabetic patients among a host of traditional cardiovascular disease (CVD) risk factors (25). However, differential development patterns of ED and CAD have not previously been investigated in a rodent model.

Impaired endothelial function appears to be due, at least in part to excess oxidative stress (8, 26), as oxidative stress in the vascular wall is a prominent feature of vascular disease (27, 28). Oxidative stress is increased in obesity (6, 12, 29, 30), thus excessive oxidative stress may link obesity to vascular disease and may be a prominent contributor to the reduction in endothelial function observed in obesity. NADPH oxidase, in particular, is considered a prominent source of vascular derived reactive oxygen species (ROS), the expression of which has shown to be elevated in arteries of human diabetes and CAD patients (31, 32). Nitric oxide (NO) is a major mediator of penile erection (33) and regulator of blood flow and vasomotor tone in the coronary and peripheral circulation (34, 35). A critical requirement for normal NO synthase (NOS)

functioning is an adequate presence of the essential co-factor tetrahydrobiopterin (BH_4) (36). A potential consequence of increased NADPH oxidase activity is oxidation of BH_4, an essential cofactor of endothelial nitric oxide synthase (eNOS) (36). Oxidation of BH_4 induces cellular BH_4 depletion, which destabilizes NOS and induces a state of "uncoupling", where NOS produces a proportionally higher amount of superoxide and lower amount of NO (36). Administration of sepiapterin stimulates intracellular BH_4 production (37, 38), providing a potential pharmacological means of reversing NOS uncoupling.

In rodent models, diets rich in saturated fat and sucrose have been used to induce aortic endothelial dysfunction (39, 40). In human populations, the Western diet has been associated with inflammation and presence of vascular adhesion molecules (41), while adipose tissue concentrations of linoleic acid have been positively associated with CAD development (42). Over the past two decades there has been motivation to replace some of the saturated fat in the American diet with polyunsaturated fatty acids (PUFA), resulting in mass consumption of vegetable oils that are rich in linoleic acid (43). Additionally, it is estimated that the percentage of daily energy intake derived from added sugars is in excess of 25% in the upper sugar consumption tertile of Americans (44, 45).

Exercise training is known to protect against cardiovascular disease (46). Four weeks of aerobic exercise training prior to bypass surgery in human CAD patients has been shown to depress internal mammary artery ROS generation, NADPH oxidase activity, $gp91^{phox}$ protein expression, as well as augment the acetylcholine-stimulated vasodilatory response. Aerobic interval training in particular has shown to produce several positive health benefits in patients with heart failure or the metabolic syndrome, including increases in aerobic capacity, flow-

mediated dilation, insulin sensitivity, as well as decreases in fatty acid synthase (47, 48). In rats bred for low running capacity, aerobic interval training has had a greater effect on improving VO_{2max}, insulin signaling, and aortic endothelial function than continuous moderate exercise training (49). Thus, aerobic interval training may be a particularly beneficial intervention at reversing or preventing endothelial dysfunction that may act through depression of NADPH oxidase activity.

The purpose of the studies of this dissertation was to investigate the impact of NADPH oxidase on obesity associated endothelial dysfunction in the peripheral vascular bed, coronary artery, and penile tissue via assessment of erectile function. Additionally, we sought to investigate the efficacy of an aerobic interval training program on prevention or reversal of these obesity associated vascular dysfunctions. As clinical studies suggest that erectile dysfunction manifests prior to detectable signs of coronary artery disease (18-21), we initially sought to investigate the time course of development of erectile dysfunction relative to the development of coronary artery endothelial dysfunction in rats fed a Western diet that is high in saturated fat and omega-6 (n-6) PUFA derived from linoleic acid, as well as simple sugars. We further sought to investigate NADPH oxidase as common mechanisms leading to Western diet associated impairment of erectile function and coronary artery endothelial function at a time point where both erectile function and coronary artery endothelial function are impaired. Additionally, we sought to develop a novel, minimally invasive technique by which to measure *in vivo* ROS production and microvascular endothelial function in peripheral vascular beds of humans, and to investigate the impact of NADPH oxidase on *in vivo* ROS production and microvascular endothelial dysfunction associated with human obesity. We further sought to investigate the impact of an 8-week aerobic interval training intervention on in vivo ROS production and

microvascular endothelial function. These studies were designed to address the following specific aims:

Aim 1) To determine if development of erectile dysfunction precedes coronary artery endothelial dysfunction in response to a Western diet.

Aim 2) To determine if augmented NADPH oxidase activity contribute to diet-induced erectile dysfunction and/or coronary artery endothelial dysfunction.

Aim 3) To determine if NADPH oxidase derived ROS contribute to peripheral endothelial dysfunction in obese individuals.

CHAPTER 2: REVIEW OF LITERATURE

Reactive Oxygen Species

Oxidative stress is traditionally used to describe situations in which the generation of oxidants overwhelms antioxidant defense systems, resulting in oxidative damage to host tissue macromolecules. Oxidative stress may result from an increased production of oxidants and/or a decrease in antioxidant defense mechanisms (50). The production of oxidants refers to the production of ROS, which are redox derivatives of oxygen. The parent molecule of the ROS cascade is the superoxide anion (O_2^-) that is formed by the addition of an electron to O_2. O_2^- is a very short lived, highly reactive molecule that can be dismutated spontaneously into the hydroperoxyl radical (HOO^-), hydrogen peroxide (H_2O_2), or peroxynitrite ($ONOO^-$), or enzymatically by superoxide dismutase (SOD) into H_2O_2 (50). ROS act as intracellular signaling molecules that modulate many biochemical processes (51), however, in the state of excessive ROS production the signaling process acts to decrease NO bioavailability and cause damage to lipids, proteins, and DNA. As a consequence, apoptosis and increased cellular permeability may occur, which lead to endothelial dysfunction, inflammation, and vascular remodeling which all contribute to cardiovascular disease pathologies (28).

Within the vasculature, NADPH oxidase, eNOS, and xanthine oxidase (XO) are considered the major sources of ROS (8, 28, 52). NADPH oxidase in particular has been shown to be the predominant producer of ROS within vascular tissue (27, 53). NADPH oxidase is a protein consisting of multiple subunits located in the cytosol ($p40^{phox}$, $p47^{phox}$, $p67^{phox}$, the small GTPase Rac) and the cellular membrane ($p22^{phox}$, $gp91^{phox}$). The membrane bound catalytic subunits $gp91^{phox}$ and $p22^{phox}$ contain the complete machinery for O_2^- production and produce low levels of O_2^- in the absence of stimulation by the cytosolic subunits (28). Complete

activation of the catalytic subunits likely involves the translocation of $p47^{phox}$ and $p67^{phox}$ from the cytosol and binding to the membrane complex, a process stimulated by the PKC-dependent serine phosphorylation of $p47^{phox}$ (54, 55). Upon activation, the membrane bound subunits utilize intracellular NADH or NADPH and transfer electrons across the membrane which reduce extracellular O_2 to O_2^- (27, 55). NADPH oxidase activity can be inhibited pharmacologically by apocynin, a catechol from the herb *Picroria kurroa* which impedes the assembly of $p47^{phox}$ and $p67^{phox}$ within the membrane complex (56), or by diphenylene iodonium (DPI) which disrupts electron flow through flavoproteins including NADPH oxidase and NOS.

Xanthine oxidases catalyze the oxidation of hypoxanthine to xanthine and xanthine to uric acid. Reoxidation of a fully reduced xanthine oxidase involves the transfer of six electrons to O_2, yielding two molecules of H_2O_2 and two molecules of O_2^- (28). Xanthine oxidases are widely distributed throughout the body, however there is a circulating form of XO that can bind to glycosaminoglycans on the surface of endothelial cells and may acquire increased stability and oxidant-producing capacity upon binding (57). Xanthine oxidase does not appear to be as significant of a source of vascular ROS as NADPH oxidase in conditions of increased oxidative stress. XO is increased in the coronary arteries of patients with CAD, but treatment with oxypurinol, a XO inhibitor does not inhibit vascular O_2^- production nearly to the extent that DPI and apocynin do (32). Treatment of cultured endothelial cells with lysophosphatidylcholine induces increased ROS generation which can be blunted by DPI but not allopurinol, a XO inhibitor (58). DPI also significantly attenuates ROS generation in endothelial cells subjected to high-glucose conditions, where oxypurinol does not (59). Similarly, excessive ROS generation in aortic segments from spontaneously hypertensive rats can be attenuated by DPI and apocynin, but not oxypurinol (60).

Nitric Oxide

Nitric oxide (NO) is the primary vasodilator produced by the endothelium which regulates blood flow and vasomotor tone in the coronary and peripheral circulation (34, 35). Inhibition of NO production reduces whole-limb blood flow and nutritive blood flow at the microvascular level (35, 61). NO is a lipid soluble gas that rapidly diffuses into the vascular smooth muscle where it binds to guanylate cyclase, resulting in an increase of guanosine 3',5'-cyclic monophosphate (cGMP) production which induces smooth muscle relaxation and vascular dilation (62, 63). NO is produced within the vasculature by endothelial nitric oxide synthase (eNOS) which converts L-arginine and O_2 to NO and L-citrulline.

eNOS is a homodimeric protein robustly expressed in endothelial cells that requires several redox-active cofactors, including nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), calmodulin, and tetrahydrobiopterin (BH_4) (64, 65). eNOS is primarily membrane bound and associated with golgi membranes and plasmalemmal caveolae (66, 67). The binding of eNOS and caveolin inhibits the enzymatic activity, which is reversed upon binding of Ca^{2+}/calmodulin (68).

eNOS is activated by an increase of intracellular Ca^{2+}, which can be induced by agonists such as thrombin, bradykinin, acetylcholine, VEGF, and insulin, or by an increase in blood flow via the subsequent increase in vascular shear stress (69-72). Activation of eNOS is also regulated by phosphorylation status, where phosphorylation at Ser^{1177}, Ser^{633}, and Ser^{614} are stimulatory, and phosphorylation at Thr^{495} and Ser^{116} are inhibitory (73). Ser^{1177} phosphorylation activates eNOS catalytic activity via inhibition of calmodulin dissociation from eNOS and enhancement of the internal rate of eNOS electron transfer (70, 74). Phosphorylation of Ser^{1177} is catalyzed by Akt, cyclic AMP-dependent protein kinase (PKA), cyclic GMP-dependent protein kinase (PKG),

AMP-activated protein kinase (AMPK), and Ca^{2+}/calmodulin-dependent protein kinase II (75). Thr^{495} phosphorylation interferes with the binding of calmodulin by eNOS, thus dephosphorylation of Thr^{495} enhances the interaction of eNOS and calmodulin (76). Protein kinase C (PKC) is most likely the kinase responsible for phosphorylation of Thr^{495}, while serine-threonine protein phosphatase I (PP1) is the most likely phosphatase that dephosphorylates Thr^{495} (65). eNOS activity is further enhanced by its association with heat shock protein 90 (Hsp90). Hsp90 undergoes tyrosine phosphorylation in response to various eNOS agonists, allowing it to associate with eNOS (77, 78). Hsp90 binding stimulates eNOS activity by enhancing the affinity of eNOS for binding calmodulin and by enhancing the interaction of eNOS with Akt which increases the rate of Akt-dependent Ser^{1177} phosphorylation (79, 80). Agonist-stimulated eNOS activation is thus induced by simultaneous changes in Ser^{1177} and Thr^{495} phosphorylation and Hsp90 binding, resulting in changes in the accessibility of the calmodulin-binding domain of eNOS to calmodulin, which displaces caveolin from eNOS to release the tonic eNOS inhibition (64, 65).

NO bioactivity is used as a practical surrogate for endothelial function, thus a reduced ability of the endothelium to produce NO is commonly the defining characteristic of endothelial dysfunction. Despite endothelial dysfunction increasing with adiposity and metabolic syndrome associated diseases (7, 8, 81), the role of the eNOS protein in this progression is unclear. eNOS mRNA has been shown to be increased in streptozotocin-induced diabetic rat glomerulus, despite a decrease in glomerular NO production (82). Similarly, eNOS protein expression has shown to be increased in cerebral microvessels of diabetic obese Zucker rats despite the cerebral arterioles of these rats exhibiting an impaired vasorelaxation response to acetylcholine and ADP (83). Additionally, eNOS protein expression increases in cultured mouse microvessel endothelial cells

(84, 85) and human aortic endothelial cells (86) when exposed to high glucose conditions despite a decrease in NO production. eNOS protein expression has also been shown to be increased in the aortic wall of $LDLR^{-/-}$ mice fed an atherogenic diet compared to a group fed a normal diet (87). Fulton et al (88) observed no change in eNOS protein expression in heart, skeletal muscle, aorta, or cardiac eNOS mRNA levels in insulin resistant obese Zucker rats compared to lean Zucker rats, in addition to no differences in cardiac eNOS Thr^{494} [Thr^{495}(hum)], or Ser^{632} [Ser^{633}(hum)] phosphorylation or eNOS-Hsp90 protein-protein interaction. An increase in Ser^{1176} [Ser^{1177}(hum)] phosphorylation was observed in OZR despite an impaired vasodilatory response to acetylcholine and reduced serum NO_2^- (88). However, both eNOS protein content and activity has been shown to be reduced in skeletal muscle homogenates of overweight humans relative to lean counterparts (89). One possible explanation for the observation of increased eNOS expression coinciding with reduced NO production or vasodilatory response to acetylcholine is the phenomena of eNOS uncoupling, with subsequent compensatory upregulation of eNOS mRNA and protein content.

ENOS Uncoupling

Under normal conditions, eNOS is a homodimeric oxidoreductase, where the reductase domain generates electron flow from NADPH through the flavins FAD and FMN that are transferred to the oxidase domain of the other monomer where L-arginine oxidation occurs at the heme group in the active site (36). A critical requirement for normal eNOS functioning is an adequate presence of the cofactor tetrahydrobiopterin (BH_4). BH_4 binds close to the heme active site at the interface between the two monomers, interacting with residues from both (90). Maintenance and stabilization of eNOS dimers is dependent on BH_4, which also plays a role in the oxidation of L-arginine and the subsequent generation of NO. When BH_4 is limited or absent,

eNOS dimerization is destabilized, leading to a reduction in the relative proportion of eNOS dimers to monomers present in the cell. Additionally, eNOS catalytic activity becomes "uncoupled", where the stoichiometric coupling between the reductase domain and L-arginine oxidation at the active site is lost. However, electron transfer from NADPH through the flavins to O_2 is not inhibited, but results in the formation of O_2^- rather than NO (36).

Uncoupling of eNOS has been demonstrated by Zou et al (91) who showed that recombinant eNOS lacking L-arginine or BH_4 does not form L-citrulline, but does exhibit Ca^{2+}/calmodulin-dependent NADPH oxidase activity. O_2^- production is markedly increased in $apoE^{-/-}$ mouse aortas, but can be attenuated by removal of the endothelium or by sepiapterin (BH_4 precursor) exposure (92). Sepiapterin exposure to these aortic rings also improves the vascular relaxation response to acetylcholine and the calcium ionophore A23187, demonstrating the endothelial dependence of sufficient BH_4 levels (92). Furthermore, the eNOS:BH_4 ratio correlates with L-NAME inhibitable O_2^- production in cells with varying BH_4 concentrations, showing a BH_4 concentration dependence for eNOS "coupling" (93). BH_4 concentrations may be modulated by synthesis or metabolism. Guanosine triphosphate cyclohydrolase-1 (GTPCH-1) catalyzes the conversion of GTP to dihydroneopterin, where BH_4 is synthesized by further steps catalyzed by 6-pyruvoyltetrahydropterin synthase and sepiapterin reductase, though GTPCH-1 appears to be the rate limiting enzyme in BH_4 synthesis (94, 95). BH_4 concentration may also be lowered by partial oxidation to dihydrobiopterin (BH_2), which goes through a BH_3^+ intermediate (96). BH_4 may then be regenerated from BH_2 by dihydrofolate reductase (DHFR) (95). Indeed, knockdown of GTPCH-1 and DHFR attenuates VEGF-induced eNOS activity and NO production, which can be recovered by BH_4 supplementation (97). Supplementation of endothelial cells with BH_2 also attenuates VEGF-induced NO production and increases ROS

production, which can be increasingly reversed by increasing concentrations of BH_4 supplementation (97). Increasing BH_2 levels cause an inhibition of dephosphorylation of eNOS at Ser^{116}, which can also be recovered by supplementation with BH_4 (97). These findings indicate that the activity state of eNOS is not just reliant on intracellular BH_4 concentrations, but may be influenced by the BH_4:BH_2 ratio which is dependent upon the cellular redox state. The vasoprotective effects of ascorbic acid may potentially be mediated through maintenance of intracellular BH_4 concentrations and stabilization of the eNOS dimer. Supplementation of cultured endothelial cells with ascorbic acid has shown to preserve eNOS acitivity and NO production by recycling the BH_3^+ radical back to BH_4 (98). Chronic oral supplementation of $apoE^{-/-}$ mice with ascorbic acid increases the aortic BH_4/BH_2 ratio, restores eNOS activity, and augments the vasodilatory response to acetylcholine (99).

Depletion of intracellular BH_4 levels in various disease states appears to be dependent upon enhanced generation of $ONOO^-$. Exposure of cultured endothelial cells to O_2^- modestly degrades BH_4, but exposure to even lower concentrations of $ONOO^-$ degrades BH_4 to a much greater extent (92). Even in the absence of overt disease, exposure of C57Blk/6 rat aortic rings to $ONOO^-$ increases O_2^- production, which can be attenuated by L-NAME, sepiapterin, or endothelial denudation (92). Similarly, exposure of BAECs to $ONOO^-$ results in reduced NO production, as well as increased O_2^- production which is inhibitable by L-NAME and BH_4 administration (96). Exposure of $apoE^{-/-}$ mouse aortic rings to uric acid, a $ONOO^-$ scavenger, attenuated O_2^- production and enhanced vascular relaxation to acetylcholine and A23187 (92). Additionally, NO production is preserved in cultured endothelial cells exposed to $ONOO^-$ in the presence of uric acid, but not SOD, supporting the hypothesis that reduced NO production is the result of eNOS uncoupling rather than inactivation by O_2^- (96). Furthermore, recombinant eNOS

subjected to increasing concentrations of $ONOO^-$ increases eNOS monomers and decreases eNOS dimers, which coincides with decreased eNOS activity, decreased L-citrulline formation, and increased NADPH oxidation (91). $ONOO^-$ exerts its action upon eNOS by oxidizing the zinc-thiolate cluster, which causes the release of zinc and consequently cleaves the dimers into monomers under reducing conditions (91).

$ONOO^-$ is formed by the combination of O_2^- and NO. NADPH oxidase appears to be the predominant enzyme that contributes O_2^- to enhanced $ONOO^-$ production within the vasculature. Vascular O_2^- production is enhanced in deoxycorticosterone acetate-salt (DOCA-salt) induced hypertensive rats (100). The enhanced O_2^- production appears to be mediated through eNOS uncoupling as O_2^- production is attenuated by L-NAME or removal of the endothelium, in addition to $eNOS^{-/-}$ mice exhibiting no increase in O_2^- production when treated with DOCA-salt (100). BH_4 content is also attenuated in DOCA-salt hypertension, however both BH_4 levels and O_2^- production are normal in DOCA-salt treated $p47^{phox-/-}$ mice, indicating that eNOS is not uncoupled by DOCA-salt hypertension in the absence of fully functioning NADPH oxidase (100). Thus, NADPH oxidase may be the O_2^- source that creates $ONOO^-$. Similarly, angiotensin II infusion into rats induces hypertension, along with an increase in aortic eNOS mRNA and protein expression and a decrease in NO production (101). The enhanced O_2^- production induced by angiotensin II is attenuated by treatment with the PKC inhibitor chelerythrine, indicating that PKC somehow contributes to vascular O_2^- production (101). These pathways have been further investigated by Xu et al (102), who found that treatment of HUVECs with hypochlorous acid (HOCl) increased O_2^- production and dose dependently decreased the eNOS dimer:monomer ratio. HOCl treatment also increased phosphorylation of PKC-ζ and translocation of $p67^{phox}$ and $p47^{phox}$ from the cytosol to the plasma membrane, all of which is abolished by PKC-ζ inhibition

(102). HOCl enhanced O_2^- production was suppressed by apocynin and abolished in $p67^{-/-}$ mice, while eNOS dimer dissociation was blocked in $p67^{-/-}$ mice and in mouse aorta by treatment with uric acid (102). Similar results have been observed by Guzik et al (31, 32) in diseased human arteries. Internal mammary arteries of diabetic patients and coronary arteries of CAD patients exhibit increased endothelial O_2^- production and an upregulation of $p22^{phox}$, $p47^{phox}$, and $p67^{phox}$ coinciding with increased NADPH oxidase activity (31, 32). The elevation in O_2^- production by diabetic vessels is inhibited by sepiapterin and chelerythrine (31). Taken together, these results indicate that upregulation of vascular O_2^- production by NADPH oxidase may be mediated by PKC, and may increase $ONOO^-$ formation in vascular disease states, and in turn lead to eNOS uncoupling through oxidation of BH_4, which reduces NO production and further increases endothelial O_2^- production.

Exercise Training

Regular exercise training is associated with increased vasodilator capacity as several longitudinal studies provide convincing evidence for the antiatherogenic effect of regular physical activity (81). Exercise has also been shown to be an effective method in which to improve endothelial dysfunction in conditions associated with obesity such as hypercholesterolemia, hypertension, Type 2 diabetes, CAD, and heart failure (81). In addition to simple prevention of atherogenesis, exercise training can improve vascular function in healthy individuals. Both continuous moderate intensity exercise and high-intensity interval training improve vasodilator capacity in popliteal arteries of healthy human subjects (103). Aortic relaxation to acetylcholine is improved 24 hours after a single bout of high-intensity interval training in healthy rats, and improves to a greater degree following six weeks of training (104). Chronic high-intensity interval training has also shown to improve VO_{2max} and aortic relaxation

to acetylcholine more than continuous moderate intensity exercise in rats bred for a low running capacity, which corresponds to a 2-fold increase in aortic eNOS protein expression (49). Chronic high-intensity interval training also has shown to improve relaxation to acetylcholine in white gastrocnemius arterioles of healthy rats, alongside increases in eNOS protein expression in the aorta, iliac artery, femoral artery and various gastrocnemius arterial branches (105). Continuous moderate intensity exercise has shown to increase eNOS protein expression in human internal mammary arteries (106), in rat aorta (107, 108), left ventricle (109) and aged rat cavernosum (110), but did not change eNOS protein expression in rat soleus muscle, however soleus eNOS activity, Ser^{1179} phosphorylation, and eNOS-Hsp90 association were all increased (111).

Improvements in vasodilator capacity resulting from aerobic exercise training may at least in part be due to reductions in oxidative stress. Four weeks of aerobic exercise prior to bypass surgery in patients with CAD has shown to improve internal mammary artery relaxation to acetylcholine and increase eNOS mRNA, protein expression, Ser^{1177}-phosphorylation/eNOS protein ratio, and Akt-phosphorylation (106). The same training stimulus has been shown to decrease internal mammary artery ROS generation, NADPH oxidase activity, and $gp91^{phox}$ mRNA and protein expression (112). Chronic endurance exercise has also shown to reduce O_2^- production and NADPH oxidase activity in $apoE^{-/-}$ mice (108), and reduce $p67^{phox}$ expression in miniature swine aortic endothelium (113) and the carotid arteries of old mice (107), while apocynin only improved vasodilation to acetylcholine in the old sedentary mice which had reduced eNOS expression and Ser^{1177}-phosphorylation. Similarly, exercise reduced aortic O_2^- production, NADPH oxidase activity, $p47^{phox}$ and $p67^{phox}$ expression in healthy mice, which corresponded with an increase in eNOS mRNA and protein expression (108). Chronic exercise training has also shown to attenuate NADPH oxidase activity and markedly augment eNOS

dimers in the left-ventricle of diabetic Goto-Kakizaki rats (109), while BH_4 supplementation has shown to augment flow-mediated dilation in old sedentary individuals, but not in old exercise trained individuals (114), suggesting that chronic exercise training may prevent eNOS uncoupling. Some of the vasoprotective effects of exercise may be mediated through the increase in vascular shear stress. Duerrschmidt et al (115) found that short term application of shear stress to HUVECs increased O_2^- formation, while long-term shear stress decreased O_2^- formation, $gp91^{phox}$ and $p47^{phox}$ mRNA and protein expression along-side an increase in NO formation and eNOS protein expression. The effects of shear stress on oxidative stress appear to be due to enhanced NO formation as application of an NO donor mimics the effects of long term shear stress resulting in decreases in O_2^- formation, $gp91^{phox}$, and $p47^{phox}$, while the downregulation of O_2^- formation, $gp91^{phox}$, and $p47^{phox}$ by long-term shear stress can be blocked by co-application of L-NAME (115). Thus, exercise may regulate NADPH oxidase derived O_2^- production by the endothelium through a shear-stress induced increase in NO bioavailability. This mechanism may contribute to the regulation of endothelial NO/O_2^- balance and the antiatherosclerotic and vasoprotective potential of shear-stress and aerobic exercise. These findings suggest that aerobic exercise may downregulate NADPH oxidase activity in endothelial dysfunction states through an increase in NO production mediated by vascular shear stress.

Erectile Function

A number of epidemiological studies indicate that obesity is a significant, independent risk factor for erectile dysfunction (ED) (14, 16, 116). In addition, abdominal obesity and a sedentary lifestyle each result in an approximately 50% greater likelihood of having ED, regardless of BMI (117). Similar to many cardiovascular risk factors, ED is more highly associated with Western-style dietary intake than Mediterranean-style dietary intake (41, 118).

Reduction in erectile function may be an early indication of cardiovascular risk, as patients with ED are twice as likely to develop major adverse cardiac events compared to those without ED (17). Recognition of reduced erectile function may be an important indicator of future adverse cardiac events, as clinical trial data suggests that the presence of ED in otherwise healthy men may be associated with early (subclinical) signs of CAD that may not be detectable during stress testing (18-21). The time interval between the onset of ED symptoms and the occurrence of CAD symptoms is estimated at 2-3 years, and an estimated 3-5 years between onset of ED and a cardiovascular event (myocardial infarction or stroke) (22-24). It is estimated that men younger than 60 with ED have a 2.3 higher probability of having CAD compared to those that do not have CAD (119). Furthermore, men with ED generally exhibit more severe CAD than those without ED, and the severity of ED may also correlate with the severity of CAD (24, 120-122). Lifestyle interventions such as diet and exercise are known to augment coronary endothelial function in CAD patients (32, 112), thus it is reasonable that such interventions may positively impact erectile function in ED patients. Indeed, Esposito et al. demonstrated that obese men given diet and activity advice both lost weight and improved self-reported erectile capacity, while improvements in erectile function correlated with improvements in metabolic profile (123). Moderate intensity treadmill running has also shown to preserve cavernosal strip relaxation to ACh (124) and NMDA induced erectile function in STZ-diabetic rats (125). The association between risk factors for the metabolic syndrome, ED and CVD is strong, and given that the prevalence of ED is projected to double by 2025 (from 1995) (126) , it is imperative that the common underlying mechanisms be identified and ED be recognized as an early and preventable risk factor for CAD. Currently it is likely that ED prevalence is higher than reported and corresponds more closely to the current prevalence of obesity. In a study where men with

obesity or at least one component of the metabolic syndrome were asked if they had erectile dysfunction, follow-up administration of the Erectile Function domain of the International Index of Erectile Function (IIEF) questionnaire revealed that of men responding "yes" 96% had ED, of men responding "unsure" 90% had ED, and of men responding "no" 36% had ED (127). This lack of recognition of ED symptoms highlights an important need for increased screening for ED to potentially unmask common underlying CAD symptoms.

Electrical field stimulation (EFS) of the major pelvic ganglion or cavernosal nerve in rats provides an *in vivo* method in which to evaluate erectile function. This method provides a means to obtain information on the quality of erections assessed as an increase in intracavernosal pressure (ICP) relative to mean arterial pressure (MAP), a method which has provided a significant amount of our knowledge of erectile function over the past two decades (128). Reduced ICP to FES has been observed in several rodent models of the metabolic syndrome, such as diabetic models induced by streptozotocin (129, 130) and alloxan (131), obese-diabetic Zucker rats (132), Goto-Kakizaki diabetic rats (133), angiotensin II supplemented (134), DOCA-salt (135), and spontaneously hypertensive rats (135, 136), as well as $ApoE^{-/-}$ mice fed a hypercholesterolemic diet (137) and $LDLR^{-/-}$ mice fed a western diet (138). Similar to endothelial dysfunction elsewhere, ROS is implicated in the pathogenesis of erectile dysfunction (139, 140). Increased oxidative stress has been observed in cavernosal tissue of several models, including streptozotocin-induced diabetes (129, 130, 141, 142), angiotensin II supplemented (134) and spontaneously hypertensive rats (143), $LDLR^{-/-}$ mice fed a western diet (138), high-fat diet fed pigs (144), and cholesterol fed rabbits (145). Furthermore, chronic anti-oxidant treatment with α-lipoic acid partially corrected cavernosal ACh-stimulated relaxation (130), while supplementation of the anti-oxidant AC3056 (141) and SOD-transfection (129) improve voltage-

dependent erectile response in STZ-diabetic rats. NADPH-oxidase induced eNOS uncoupling presents an attractive hypothesis for the source of elevated ROS/reduction in erectile function. Exposure of cavernosal strips to apocynin improves both relaxation to an ACh analog and attenuates ROS production from hypercholesterolemic rabbits, while allopurinol and rotenone were ineffective (145). Similarly, drinking-water supplementation of apocynin blunts the elevated cavernosal $p47^{phox}$ content and oxidative stress in hypertensive (134) and hypercholesterolemic (138) models and restores the eNOS dimer/monomer ratio (138). Additionally, oral supplementation of BH_4 to men with mild ED improved erectile response to visual stimulation (146), while four days of sepiapterin injection improves voltage-dependent erectile response in aged rats (147). Furthermore, there is reason to believe that this mechanism of attenuated erectile function can be prevented by exercise training. Treadmill running of high-fat diet fed pigs preserved cavernosal cGMP and eNOS dimer/monomer ratio compared to their sedentary high-fat diet counterparts (144).

Consistent with the hypothesis that erectile dysfunction is an early indicator of future CAD, it is likely that a reduction in erectile dysfunction occurs prior to the onset of coronary endothelial dysfunction (132, 148, 149). In 17-18 week old lean (LZR) and obese Zucker rats (OZR), there is a 4-fold increase in the media:lumen ratio in the penile arteries of OZR compared to LZR, where no difference was observed in the coronary arteries (149). This finding indicates that atherosclerotic development occurs in the penile artery prior to the coronary artery, and reflects the state of endothelial function as the vasodilatory response to ACh in the OZR is attenuated in the penile artery but not the coronary artery (149). Oltman et al. (148) found similar results as there was no difference in the coronary vasodilatory response to ACh between LZR and OZR at 16-24 weeks of age, however this response was attenuated in OZR at 28-36 weeks of

age. It is also likely that the reduction in penile artery endothelial dysfunction corresponds to erectile dysfunction as Wingard et al. (132) observed a reduction in ICP/MAP in OZR at 16-20 weeks. These findings in Zucker rats may translate to other models of obesity. Ten weeks of high-fat diet attenuates ACh-stimulated vasodilatory response in the penile artery of Wistar rats (150), but not the coronary artery (151). However, there is likely a reduction in endothelial derived NO production and an alteration of the smooth muscle response to NO in the coronary arteries. The coronary ACh-stimuated vasodilatory response was significantly attenuated by L-NAME treatment in lean but not obese animals (151). Additionally, the vasodilatory response to SNP is augmented in the obese animals (151). This obesity-related compensation by smooth muscle is likely present in the penile artery as ACh stimulates dilation of the penile artery in the presence of a NOS inhibitor in obese animals but not lean animals (150).

Diet-Induced Obesity

Several studies have demonstrated impaired endothelial function in diet-induced obese models in aortas of rats (39, 40, 152-154), mice (155), and coronary arteries of pigs (6, 156). Similar to obese Zucker rats, augmented sensitivity to NO has been demonstrated in diet-induced obese rat aortas (40, 153) and in a study where ACh-induced NO production was attenuated in coronary arteries despite no change in ACh-induced coronary flow in diet-induced obese dogs (157). There is indication that increased oxidative stress, increased NADPH oxidase activity and reduced eNOS activity contribute to endothelial dysfunction in this model. Attenuated eNOS content has been observed in aorta (39, 40, 152), heart (40), kidney (40), and gastrocnemius (39), along with attenuated aortic eNOS phosphorylation (154). In addition, eNOS content is attenuated in the cavernosum of diet-induced obese rats, which is restored with metformin treatment (158). Aortic $gp91^{phox}$ (153), $p47^{phox}$ (152), and cardiac $p22^{phox}$ (159) expression are

elevated in diet-induced obesity and may be blunted by antioxidant treatment (152, 159). Elevated oxidative stress has been documented in this model by increased aortic nitrotyrosine (39, 152) and reversibility of endothelial dysfunction with SOD incubation (155). Additionally, endothelial dysfunction following diet-induced obesity appears to be reversible with the addition of exercise training and/or diet modification (154, 156).

Summary

Evidence has been provided in the above sections that oxidative stress in obesity may be a significant contributor to the reduction in nitric oxide bioavailability and ensuing vascular dysfunction often observed in conditions associated with obesity. Under this paradigm, vascular ROS is induced by an upregulation of NADPH oxidase activity which contributes to enhanced production of $ONOO^-$, potentially leading to BH_4 depletion by oxidation and dissociation of eNOS dimers into monomers. This situation results in a reduction in NO production and increased eNOS dependent O_2^- production which further contribute to vascular oxidative stress and endothelial dysfunction. Current evidence suggests that endothelial dysfunction manifest as erectile dysfunction, peripheral endothelial dysfunction, or coronary endothelial dysfunction is prevalent in obesity and several models of the metabolic syndrome. It remains likely that these manifestations of endothelial dysfunction are linked through similar mechanisms, of which NADPH oxidase induced eNOS uncoupling is an attractive hypothesis. Chronic aerobic interval training, however, has the potential to ameliorate vascular oxidative stress and the ensuing endothelial dysfunction in obesity.

CHAPTER 3: METHODS

Animal Studies

Experimental Animals and Diets

Male Sprague-Dawley rats were purchased at 5, 9, or 13 weeks of age (Charles River Laboratories, Wilmington, MA) and housed in the Department of Comparative Medicine, in pairs when possible, in a temperature controlled room (22 ± 1°C) with a 12h:12h light dark cycle. For aim 1, rats were fed a Western diet (WD) (Teklad Diets 110365, Harlan Laboratories, Madison, WI) for the final 4 (n = 8), 8 (n = 8), or 12 (n = 5) weeks of life, or a control diet (Teklad Diets 110367, Harlan Laboratories, Madison, WI) for the final 8 (n = 5) weeks of life. For aim 2, rats were fed the Western diet (n = 11) or control diet (n = 12) while remaining sedentary for 12 weeks, or the Western diet (n = 11) or control diet (n = 8) through the duration of a 12 week exercise intervention. The Western diet was designed to be high in fat and sucrose, with an equal proportion of saturated fatty-acids (SFA) and PUFA, with a high proportion of n-6 PUFA derived from linoleic acid. The control diet was designed to be a normal-fat, normal-carbohydrate diet with equivalent levels of vitamins, minerals, and protein to the Western diet when considered on a basis of kcal density (Tables 1, 2). All rats were sacrificed at 19-20 weeks of age. All procedures were performed in accordance with the Guiding Principles in the Care and Use of Animals established by the National Institute of Health and approved by the Institutional Animal Care and Use Committee.

Table 1 Composition of the Western diet and Control diet.

	Western Diet	Control Diet
	g/kg	g/kg
Casein	195	155
L-Cystine	3	2.3
Sucrose	340	150
Corn Starch	62.46	445.19
Maltodextrin	60	100
Anhydrous Milkfat	120	26
Soybean Oil	30	18
Safflower Oil	80	6
Cellulose	50	50
Mineral Mix, AIN-93M-MX	44	35
Vitamin Mix, AIN-76A	12.5	10
Choline Bitartrate	3	2.5
TBHQ	0.04	0.01

TBHQ, tert-butylhydroquinone.

Table 2 Macronutrient composition of the Western and control diets.

	Western Diet	Control Diet
kcal/g	4.68	3.67
Fat, % by weight	23.2	5.2
Carbohydrate, % by weight	47.6	66.3
Protein, % by weight	17.3	13.7
Fat, % of kcal	44.6	12.7
Carbohydrate, % of kcal	40.7	72.4
Protein, % of kcal	14.8	15
SFA, % of total fatty acids	39	40
MUFA, % of total fatty acids	24	26
PUFA, % of total fatty acids	37	33
C18:2 linoleic, % by weight	8.2	1.5
C18:3 linolenic, % by weight	0.3	1.57
n-6 to n-3	27	9.6
Vitamin E, IU/kg	62	50

SFA, saturated fatty acid; MUFA, mono-unsaturated fatty acid; PUFA, poly-unsaturated fatty acid

Exercise Intervention

Rats were acclimated to exercise by walking on a treadmill at 15 m/min at 0° inclination for 10 min/day for seven consecutive days prior to induction of the training/dietary intervention. The training protocol consisted of a maximal work capacity test at the beginning of each week, followed by aerobic interval training four days/week, for a total of five exercise days/week. The maximal work capacity test consisted of a 20-minute warm-up at ~50% VO_{2max}, followed by incremental increases in speed of 2.0 m/min every two minutes until volitional fatigue, followed by at 10-minute cool-down at ~50% VO_{2max}. The aerobic interval protocol consisted of a 10-minute warm-up followed by eight intervals, each consisting of a four-minute high-intensity phase and three-minute low-intensity phase, followed by a four-minute cool-down as detailed in Table 3. Running intensity increased each week until a plateau was reached in maximal work capacity at week 9. Rats were encouraged to run primarily by compressed air, with every effort made to minimize the use of the electric shock grid at the back of the treadmill belt. This protocol was designed to approximate the intensities utilized in previous studies which demonstrate the beneficial impact of aerobic interval training on endothelial function and aerobic capacity relative to continuous moderate intensity exercise protocols (49, 160, 161).

Table 3 Treadmill aerobic interval training protocol. Treadmill speed was changed at the indicated time point.

Week	1	2	3	4	5	6	7	8	9	10	11	12
Incline	17°	17°	17°	17°	17°	17°	25°	25°	25°	25°	25°	25°
Minute	Speed (m/min)											
0	10	12	14	14	15	16	14	14	14	14	14	14
10	19	20	21	22	23	24	21	22	22	22	22	22
14	14	15	16	17	18	19	16	17	17	17	17	17
17	19	20	21	22	23	24	21	22	22	22	22	22
21	14	15	16	17	18	19	16	17	17	17	17	17
24	19	20	21	22	23	24	21	22	22	22	22	22
28	14	15	16	17	18	19	16	17	17	17	17	17
31	19	20	21	22	23	24	21	22	22	22	22	22
35	14	15	16	17	18	19	16	17	17	17	17	17
38	19	20	21	22	23	24	21	22	22	22	22	22
42	14	15	16	17	18	19	16	17	17	17	17	17
45	19	20	21	22	23	24	21	22	22	22	22	22
49	14	15	16	17	18	19	16	17	17	17	17	17
52	19	20	21	22	23	24	21	22	22	22	22	22
56	14	15	16	17	18	19	16	17	17	17	17	17
59	19	20	21	22	23	24	21	22	22	22	22	22
63	14	15	16	17	18	19	16	17	17	17	17	17
66	10	12	13	14	15	16	13	14	14	14	14	14
70	stop											

Glucose, Insulin, and Lipid Profiles

All rats were fasted overnight prior to body composition studies. Approximately 0.3 ml of blood was drawn from the tail vein prior to anesthesia, from which fasting glucose concentration was measured from whole blood (Accu-Check, Roche, Basel, Switzerland). Blood was centrifuged for 20 min (4°C, 14,000 rpm), and plasma separated and frozen in a -80°C freezer until analyzed for insulin concentration with a rat/mouse insulin assay kit (Millipore, Billerica, MA). The homeostatic model of insulin resistance (HOMA-IR) was calculated from fasting glucose and insulin concentrations as HOMA-IR = [Insulin (mU/l) * Glucose (mmol/l)]/22.5 (162). Approximately 1.5 ml of blood was drawn through a splitter in the PE tubing upon placement and stabilization of the carotid cannula, at the onset of the erection assessment surgery, subsequently centrifuged and serum was separated and frozen until analyzed. Serum total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglycerides were measured with a clinical mass analyzer (UniCel DxC 600, Beckman Coulter, Indianapolis, IN).

HNE adducts

The relative amount of serum 4-hydroxynonenal (HNE)-adducts was determined by a modified ELISA approach. A standard curve of HNE-adducts was established by incubating HNE with pre-determined concentrations of bovine serum albumin (BSA). Following an overnight incubation at 37°C, HNE-BSA adducts were added to an Immunolon-coated 96-well assay plate (ThermoScientific, Rochester, NY, USA), along with diluted serum (1:40 in PBS). Samples were incubated overnight at 4°C, and subsequently washed with PBS+0.05% Tween-20 and blocked for 2 hours with NB4025 (NOF Corp., Tokyo, Japan). Samples were then incubated

with anti-HNE antibody (1 µg/ml in PBS, Oxis Research, Beverly Hills, CA, USA), for 2 hours at 37°C. Samples were washed with PBS+0.05% Tween-20 and incubated with secondary antibody for 2 hours at 27°C (goat anti-mouse HRP, Bio-Rad, Hercules, CA, USA). Following this incubation, samples were washed as before and incubated with tetramethylbenzene (TMBZ) for 20 minutes. Reactions were quenched with 1M sulfuric acid, and the absorbance of the samples at 450 nm was determined.

Anthropometrics and Body Composition

Body weight was measured prior to surgery with a triple beam balance. Lean mass and fat mass were measured immediately prior to surgery by nuclear magnetic resonance-magnetic resonance imaging (NMR-MRI) (EchoMRI 700, Echo Medical Systems, Houston, TX). Body fat percentage was calculated as ($mass_{fat}$ / ($mass_{lean}$ + $mass_{fat}$)).

Erectile Response Measurements

All rats were fasted overnight prior to erectile function studies. Rats were anesthetized with an intraperitoneal injection of 90 mg/kg ketamine and 10 mg/kg xylazine, and supplemented with an intramuscular injection as needed. The left carotid artery was cannulated with a 20-gauge needle connected via polyethylene (PE) tubing to a pressure transducer, anti-coagulated with 2.0 U/ml heparinized saline allowing for continuous measurement of systemic blood pressure and intra-arterial administration of saline. The shaft of the penis was freed of skin and fascia and the right corpus cavernosum cannulated with a 27-gauge needle connected via PE tubing to a pressure transducer, and the left corpus cavernosum cannulated with a 27-gauge needle connected via PE tubing to a syringe containing heparinized saline. The abdominal cavity was opened and the right major pelvic ganglion (MPG) exposed. Platinum bipolar electrodes attached

to a Grass Stimulator (Grass S09, Grass Technologies, West Warwick, RI) were positioned near the MPG on the cavernosal nerve to deliver electrical field stimulation (EFS), consisting of a series of 1-5 volts for 30 seconds at each voltage, delivered in 5-millisecond pulses at a frequency of 13 Hz (132). Mean arterial pressure (MAP) and intracavernosal pressure (ICP) were recorded throughout each voltage series with LabChart 7 software (ADInstruments, Sydney, Australia). Three voltage series' were conducted and averaged as the erectile response. ICP returned to baseline between each series. Following the three voltage series', either 12 µl of 10 µM sepiapterin (Cayman Chemical, Ann Arbor, MI) dissolved in heparinized saline, or 12 µl of 1 mM apocynin was injected into the left corpus cavernosum. A voltage series was conducted 30-minutes post-injection for sepiapterin, or 20-minutes post-injection for apocynin where treatment effects were maximal.

Coronary Artery Endothelium-Dependent and -Independent Vasodilation

Immediately following the erection surgery, rats were euthanized by exsanguination and double pneumothorax. Hearts were excised and four segments (0.3 – 0.8 mm) of the left anterior descending coronary artery were carefully dissected and freed from adhering myocardium. Two, 40 µm diameter, stainless steel wires were passed through each arterial segment and mounted in a multi wire myograph (DMT 620 M, Aarhus, Denmark). Vessel segments equilibrated for 45 minutes bathed in physiological saline solution (PSS) at pH 7.4, 37°C, gassed with medical grade air as described (132). The relationship between passive wall tension and internal circumference was determined for each segment, from which the internal circumference (L_{100}) corresponding to a transmural pressure of 100 mmHg for a relaxed vessel *in situ* was calculated. Vessels were set to an internal circumference equal to 0.9 times L_{100}, at which tension development is maximal in

these arteries (163). Vessels were allowed to equilibrate for another 45 minutes, and then challenged with 109 mM K^+ physiological saline solution (KPSS) to test vessel viability. Vessels were depolarized for 10 minutes and relaxed with repeated washes of PSS at 10-minute intervals. Vessels were pre-constricted with 3.0 μM 5-hydroxytryptamine (5-HT), and endothelial function tested with cumulative doses of acetylcholine (ACh) (0.001 – 10.0 μM). Following 30 minutes of repeated washes of PSS at 10 minute intervals, vessel segments were incubated in either 300 μM apocynin or 10 μM sepiapterin for 30 minutes, and the ACh cumulative dose response following 5-HT pre-constriction was repeated. Vessels were washed for 30 minutes with repeated washes of PSS at 10 minute intervals, and endothelium-independent relaxation was tested with cumulative doses of sodium nitroprusside (SNP) (0.0001 – 1.0 μM) following 5-HT pre-constriction.

Data and Statistical Analysis

The erectile response was calculated from the ratio of the average ICP divided by the average MAP during the final 25 seconds of each 30-second voltage stimulation level. The area-under-the-curve (AUC) of all voltages for each voltage series was calculated by GraphPad Prism (Prism 5.0, GraphPad, San Diego, CA). Coronary artery endothelium-dependent and -independent vasodilation was assessed by the average stress produced by each vessel in the final 30 seconds of each dose. Vessel stress was calculated by subtracting basal vessel force produced from the force produced at each respective concentration and normalized to vessel surface area. The percent relaxation of 5-HT induced constriction at each concentration was calculated as: $((1-(stress_{concentration} / stress_{5\text{-}HT}))*100)$. The relaxation responses from the four vessel segments were averaged for each rat, and analyzed as a single observation. Concentration-response curves were

generated against the logarithm of each concentration, and fit by non-linear regression to generate EC_{50} values to describe each curve (GraphPad Prism v. 5.0). Data were presented as mean ± SEM. Statistical analyses were performed with GraphPad Prism v. 5.0 or SPSS v. 19.0 (IBM, Armonk, NY, USA) with an alpha level of 0.05. Statistical differences between groups were determined by one-way ANOVA with Tukey's multiple comparisons post-hoc analysis for serum markers, EC_{50} values, and AUC values. Statistical differences between groups for the voltage-dependent erectile response, and concentration-response curves at each voltage or concentration were determined by two-way repeated measures ANOVA with Bonferroni's post-hoc analysis. Trends were determined by one-way ANOVA with a linear trend post-test in GraphPad Prism v. 5.0.

Human Study

Subjects

Young (age 18-41) men and women from Pitt County, a rural community of approximately 100,000 residents, were recruited to participate in this study. Subjects were recruited across a range of BMI from 18-40 kg/m^2. Based on pilot data (Figure 11), subjects were divided into tertiles based on BMI. Divisions of lean and non-lean groups or of lean, overweight, and obese groups, were not made because those groupings were not congruent with the pilot data (Figure 11). Tertiles based on BMI were utilized to investigate group differences in microvascular endothelial function where ROS production was elevated. Tertile 1 included subjects with a BMI range of 18.6-25.0 kg/m^2 (n = 7), tertile 2 included a BMI range of 28.3-32.5 kg/m^2 (n = 8), and tertile 3 included a BMI range of 33.0-40.4 kg/m^2 (n = 7). Prior to initiation of the study, subjects were not involved in any physical activity program (> 4 METS

for > 30 minutes per day, > 1 day per week) for the past six months. All subjects were non-smokers with no known history of cardiovascular disease. Subjects were excluded if they demonstrated a fasting blood glucose concentration greater than 125 mg/dl. Subjects were not taking any medications for hypertension, hypercholesterolemia, insulin resistance or non-insulin dependent diabetes mellitus. Subjects abstained from antioxidant supplementation for the duration of the experimental period. All female subjects were on oral contraceptive medication. There were no restrictions with regard to race, gender, or socioeconomic status. All procedures were approved by the University and Medical Center Institutional Review Board of East Carolina University.

Anthropometrics

Height was measured with a stadiometer to the nearest 0.1 cm. Body mass was measured with a digital electronic scale to the nearest 0.05 kg. BMI was calculated as body mass in kilograms divided by height in meters squared (kg/m^2). Body fat percentage was determined using dual-energy x-ray absorptiometry (DXA; GE Lunar Prodigy Advance, Madison, WI, USA).

Fasting blood glucose, insulin, and lipid profiles

Approximately 12 ml of blood was collected from an antecubital vein following an overnight fast in glass evacuated blood collection tubes (BD Medical Supplies, Franklin Lakes, NJ, USA). Blood samples were allowed to clot and then were centrifuged at 3300 rpm for approximately 10 minutes. Serum glucose, insulin, triglycerides (TG), total-cholesterol (TC), and high-density lipoprotein cholesterol (HDL-C) were assessed by a commercial clinical laboratory (Laboratory Corporation of America). Low density lipoprotein cholesterol (LDL-C) was

calculated by the Friedewald formula (164): LDL-C (mg/dl) = TC – HDL-C – (TG/5). The homeostatic model of insulin resistance (HOMA-IR) was calculated from fasting glucose and insulin concentrations as HOMA-IR = [Insulin (mU/l) * Glucose (mmol/l)]/22.5 (162).

Insertion of microdialysis probes

Three microdialysis probes were inserted into the left vastus lateralis under sterile techniques as previously described (61, 165), while the subject is resting in a hospital bed. Following administration of local anesthesia (1 ml of 1% Lidocaine HCl) above the muscle fascia, an 18-gauge catheter (Jelco, Smiths Medical, Southington, CT, USA) surrounded by plastic introducer tubing was inserted into the vastus lateralis for each probe insertion. The catheters were then withdrawn, while the introducer tubing was left in the muscle. A microdialysis probe (CMA 20 Elite, CMA Microdialysis AB, Solna, Sweden) was then inserted into each of the three introducers. The splittable introducer tubing was then pulled out of the thigh, leaving the probes in place in the muscle. The distal 10 mm of the microdialysis probe contains a semi-permeable membrane allowing for bi-directional diffusion of small molecules (< 20 kDa) and interaction of the perfusate with the local environment (< $1cm^3$). All probes were inserted at least 3 cm apart. Probes are then perfused with a 0.9% saline solution containing 5 mM ethanol (EtOH) with microinfusion pumps (CMA 107, CMA/Microdialysis, Solna, Sweden) at a flow rate of 2.0 µl/min for the remainder of the experiment. Probes were perfused for 60 minutes to allow for recovery from trauma induced by probe insertion. Following recovery from trauma, three-10 minute samples were collected from each probe in 150 µl polyethylene collection vials, which were capped and stored for analysis of baseline blood flow rates later that day. One probe had 300 µM apocynin added to the perfusate for the duration of the experiment

to test the impact of NADPH oxidase on ROS production and microvascular endothelial function (Table 4).

In vivo ROS production

Fluorescence spectrometry of skeletal muscle extracellular ROS levels was performed using Amplex Ultrared (Molecular Probes, Eugene, OR, USA). Amplex Ultrared is a fluorogenic substrate with a very low background fluorescence which reacts with H_2O_2 with a 1:1 stoichiometry to produce the highly fluorescent resorufin. 100 µM Amplex Ultrared and 1.0 U/ml horseradish peroxidase (HRP; Sigma Aldrich, St. Louis, MO, USA) were added to the perfusate allowing H_2O_2 produced *in vivo* to cross over the membrane and react with the fluorogenic substrate within the microdialysis probe. Three-20 minute dialysate samples were collected in 150 µl collection vials wrapped in aluminum foil, and fluorescence intensity was measured immediately upon collection by a TD-700 laboratory fluorometer (Turner Designs, Sunnyvale, CA, USA) at an excitation wavelength of 550 nm and an emission wavelength of 570 nm. 10 U/ml SOD (Sigma Aldrich, St. Louis, MO, USA) was then added to the perfusate allowing for the conversion of superoxide that crosses over the membrane to H_2O_2, which reacts with the fluorogenic substrate. One of the microdialysis probes contained 300 µM apocynin in the perfusion mixture (Table 4), allowing for the contribution of NADPH oxidase to ROS production to be assessed. Relative fluorescence units were converted to [H_2O_2], based on an [H_2O_2] standard curve conducted by addition of known concentrations of H_2O_2 to each perfusate following each experiment.

Microvascular endothelial function

Following assessment of ROS production, the perfusion media was changed on all three probes (Table 4). The new perfusates were composed of a 0.9% saline solution with 5 mM EtOH and one of the following three pharmacological agents: 1) 50 mM sodium nitroprusside to test the endothelium-independent vasodilatory response, 2) 50 mM acetylcholine to test the endothelium-dependent vasodilatory response, or 3) 50 mM acetylcholine + 300 µM apocynin to test the influence of NADPH oxidase on the endothelium-dependent vasodilatory response. Probes were perfused for 30 minutes to allow for equilibration, and three-10 minute samples were collected over the subsequent 30 minutes where the pharmacological response is maximal. Samples were stored in capped collection vials until analysis later that day. During perfusion, EtOH diffuses from the microdialysis membrane and away from the local area by the microcirculatory blood flow in the immediate vicinity of the microdialysis probe membrane. EtOH concentrations of the perfusate and dialysate were analyzed using an enzymatic-fluorometric method based on the conversion of EtOH and NAD to acetaldehyde and NADH, as described (166). 2.0 µl of perfusate, dialysate, or standard were added to a reaction buffer consisting of 74 mM sodium pyrophosphate, 60 mM hydrazine sulfate, 22 mM glycine, and 500 µM NAD. The reaction was then catalyzed by addition of 33.55 mU alcohol dehydrogenase. Samples were incubated in the dark for 60 minutes, and fluorescence was measured at excitation of 360 nm and emission of 415 nm. EtOH concentration was calculated based on an EtOH standard concentration curve. All samples and standards were measured in triplicate. The EtOH outflow-to-inflow ratio was calculated as [EtOH]dialysate/[EtOH]perfusate, which is inversely related to local blood flow in a non-linear fashion, and was converted to blood flow units ($ml \bullet min^{-1} \bullet 100\ g^{-1}$) using the equations of Wallgren et al (167).

Table 4. Microdialysis perfusion protocol

	Equilibrate 0-60 min	Phase 1 (3 samples) 60-90 min ‡	Phase 2 (3 samples) 90-150 min	Phase 3 (3 samples) 150-210 min	Equilibrate 210-270 min	Phase 4 (3 samples) 270-300 min ‡
Probe 1	Control	Control	Control‡	Control‡	SNP	SNP
Probe 2	Control	Control	Control*	Control**	ACh	ACh
Probe 3	Apocynin	Apocynin	Apocynin*	Apocynin**	ACh + Apocynin	ACh + Apocynin

* 100 µM amplex ultrared, 1 U/mL HRP added to perfusate to measure in vivo H_2O_2 production within the microdialysis probe.

** 100 µM amplex ultrared, 1 U/mL HRP, 10 U/mL SOD added to perfusate. SOD will convert $O_2^{\cdot-}$ that crosses the membrane into H_2O_2, which will react with amplex ultrared within the microdialysis probe. The difference in ROS between phases 2 and 3 is attributed to in vivo $O_2^{\cdot-}$ production.

‡ Ethanol o/i ratio measured to assess microvascular nutritive blood flow.

Aerobic interval training

Following the initial microdialysis session, subjects performed a standardized maximal exercise test to assess maximal aerobic capacity. A facemask was placed on the subject's face to allow for expired O_2 and CO_2 to be continuously monitored via open circuit spirometry (TrueMax 2400; Parvomedics; Salt Lake City, UT, USA). VO_{2peak} was assessed using the Storie protocol (Figure 1), a treadmill ramp protocol where the speed or incline is increased every two-minutes until volitional fatigue is reached. Heart rate was recorded every minute throughout the test and immediately upon fatigue to determine the maximum heart rate (HR_{max}). Subjects then performed an aerobic interval training intervention, consisting of exercise three days per week for eight weeks. Exercise consisted of walking/running up an incline (7-12%) on a motor-driven treadmill. Subjects performed a 10-minute warm-up at ~60% HR_{max}, followed by four 4-minute intervals at 88-92% HR_{max} interspersed by 3-minute active recovery periods at ~70% HRmax. The intervals were followed by a 4-minute cool down at ~60% HR_{max}, yielding a total exercise time of 42 minutes per session. HR was monitored throughout each training session, and intensity (speed or incline) was increased when HR failed to reach at least 88% HR_{max} during the high-intensity interval. The fasting blood draw and microdialysis session was repeated two-days following the final training session to assess the impact of the aerobic interval training intervention on extracellular ROS production and microvascular endothelial function. The maximal exercise test and anthropometric tests were repeated following the final microdialysis session to test the impact the exercise intervention on maximal aerobic capacity and body composition.

Table 5. Storie treadmill test

Subject: _____ Age: _____ Date:_____

Medications_____

Age-Predicted Maximum HR 100% _____ 85%_____

Sitting: HR _____ BP_____/_____/_____ Standing: HR _____ BP _____/_____

STAGE (min.)	SPEED (mph)	GRADE (%)	PRED. METS	HR	BP	RPE	COMMENTS (signs/symptoms)
0	3.0	0	3.0	____			_____
1	3.0	0	3.0	____	____/_____	____	_____
2	3.0	5	5.0	____			_____
3	3.0	5	5.0	____	____/_____	____	_____
4	3.0	7.5	6.0	____			_____
5	3.0	7.5	6.0	____	____/_____	____	_____
6	3.0	10.0	7.0	____			_____
7	3.0	10.0	7.0	____	____/_____	____	_____
8	3.4	10.0	8.0	____			_____
9	3.4	10.0	8.0	____	____/_____	____	_____
10	3.4	12.0	9.0	____			_____
11	3.4	12.0	9.0	____	____/_____	____	_____
12	3.8	12.0	10.0	____			_____
13	3.8	12.0	10.0	____	____/_____	____	_____
14	4.0	12.0	11.0	____			_____
15	4.0	12.0	11.0	____	____/_____	____	_____
16	5.0	9.5	12.0	____			_____
17	5.0	9.5	12.0	____	____/_____	____	_____
18	5.5	9.5	13.0	____			_____
19	5.5	9.5	13.0	____	____/_____	____	_____
20	5.9	9.5	14.0	____			_____
21	5.9	9.5	14.0	____	____/_____	____	_____
22	6.4	9.5	15.0	____			_____
23	6.4	9.5	15.0	____	____/_____	____	_____

Comments:

Data and statistical analyses

Subjects were divided into tertiles based off of BMI. Statistical differences between tertiles for metabolic parameters, ROS production, and ACh- or SNP-stimulated blood flow were assessed by one-way ANOVA with a Newman-Keuls post hoc analysis. Treatment effects of apocynin were determined with a two-way repeated measures ANOVA with a Bonferroni post hoc analysis. Differences in response to apocynin treatment across tertiles were determined with tertile by apocynin interaction effects of a two-way repeated measures ANOVA. Due to a drop-out or incompletion rate of 59% during the exercise intervention, tertiles 1 and 2 were combined because they were not different prior to training with respect to any main outcome measure. For all pre-to-post training comparisons, only data of subjects that completed the training program were included in the pre-training values. Training induced changes in metabolic parameters were determined within each group by a paired t-test. Exercise training induced changes in ROS production, ACh-stimulated blood flow, and SNP-stimulated blood flow within each group were determined by a paired t-test. Training induced changes in the effects of apocynin treatment were determined with a two-way repeated measures ANOVA. Due to low statistical power for exercise training effects, the effect size of all main outcome measures were calculated as: $[(Mean_1 – Mean_2)/\text{pooled standard deviation}]$. Effect sizes > 0.5 were considered medium effect sizes. Effect sizes > 0.8 were considered large effect sizes. Statistical analyses were performed with GraphPad Prism v. 5.0 or SPSS v. 19.0 with an alpha level of 0.05.

CHAPTER 4: RESULTS

Animal Study: Aim 1

Metabolic Parameters

There were no statistically significant increases in body weight or composition in response to the WD (Table 6). Fasting blood glucose was significantly elevated in rats fed the WD for 12 weeks compared to all other groups. Despite increased glucose, no significant differences existed for insulin or HOMA-IR between groups (Table 6). There were no apparent differences in serum lipid profiles between the control diet and WD groups (Table 6). There was a trend for increasing serum HNE adducts with increasing diet duration ($P = 0.028$), suggesting increased lipid peroxidation and systemic oxidative stress in the 8-week and 12-week WD fed rats (Table 6).

Table 6 Aim 1 Metabolic Parameters.

	Control	4-week	8-week	12-week	ANOVA P-value
Body Weight (g)	443 ± 12.2	478 ± 8.0	453 ± 13.6	489 ± 12.8	0.059
Fat Mass (g)	50.0 ± 1.59	56.5 ± 6.0	67.8 ± 10.9	73.4 ± 4.2	0.226
Lean Mass (g)	329 ± 10.8	355 ± 2.9	326 ± 7.8†	350 ± 10.3	0.018
Body Fat %	13.2 ± 0.47	13.6 ± 1.25	19.9 ± 2.10	17.3 ± 0.94	0.241
Glucose (mg/dl)	115 ± 2.9	105 ± 4.8	110 ± 3.3	131 ± 3.5*,†,‡	0.001
Insulin (pmol/l)	126.7 ± 33.4	87.1 ± 15.0	159.8 ± 30.4	155.8 ± 14.0	0.320
HOMA-IR	5.06 ± 1.36	3.19 ± 0.66	6.03 ± 1.09	7.02 ± 0.62	0.207
Triglycerides (mg/dl)	52.3 ± 14.9	45.9 ± 14.1	38.8 ± 6.95	28.8 ± 6.9	0.597
Cholesterol (mg/dl)	32.3 ± 3.47	27.9 ± 2.96	38.8 ± 2.76†	31.4 ± 1.72	0.059
HDL-C (mg/dl)	22.4 ± 2.78	21.6 ± 1.61	30.9 ± 2.63†	24.9 ± 1.40	0.020
LDL-C (mg/dl)	7.0 ± 0.45	7.7 ± 0.86	8.5 ± 0.24	8.7 ± 0.55	0.202
HNE-adducts	0.695 ± 0.09	0.752 ± 0.23	0.976 ± 0.14	1.20 ± 0.15	0.095

Values are means ± SEM, N = 3-8 animals per group. * $P < 0.05$ vs. Control, † $P < 0.05$ vs. 4-week, ‡ $P < 0.05$ vs. 8-week.

Voltage-Dependent Erectile Response

Erectile function was assessed by measuring the AUC of the voltage-dependent erectile response, suggestive of the ability to achieve and maintain an erection upon electrical field stimulation. The AUC was significantly depressed following 8 weeks of the Western diet, and remained similarly depressed following 12 weeks of the Western diet (Figure 1A). The ICP/MAP in response to the intermediate voltages (2V and 3V) of stimulation were significantly attenuated following 8 and 12 weeks of the WD (Figure 2). Intracavernosal treatment with sepiapterin had no effect on the voltage-dependent erectile response of control diet rats, but significantly augmented the erectile response of all groups of WD fed rats (Figure 3). The ICP/MAP following sepiapterin treatment was increased in response to 3V of stimulation was increased in 4-week (Figure 3B) and 8-week (Figure 3C) WD fed rats, and in response to 4V of stimulation in 12-week WD fed rats (Figure 3D).

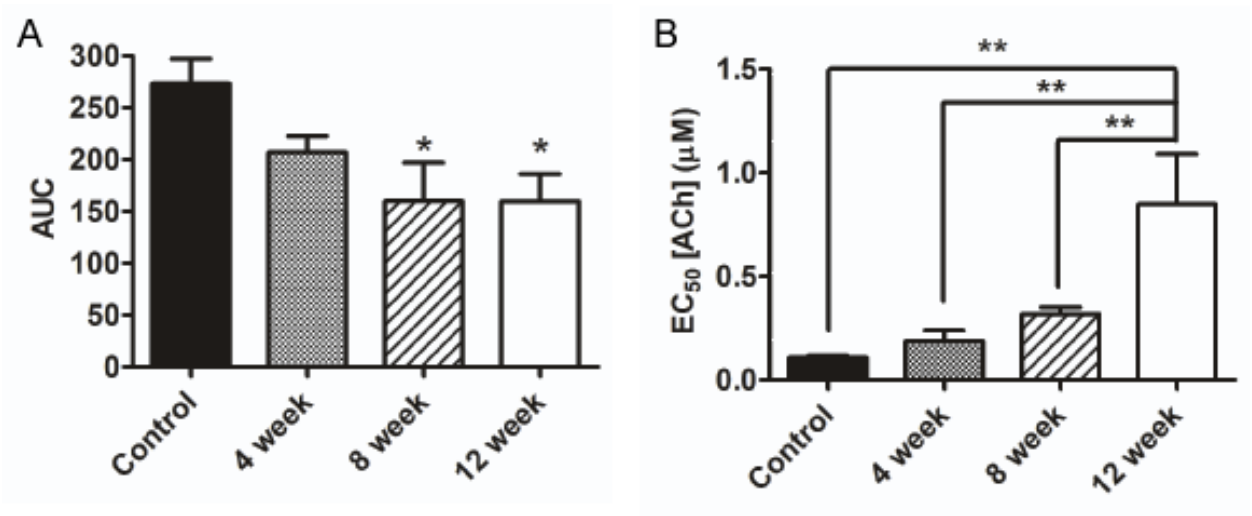

Figure 1 Rats developed erectile dysfunction prior to coronary artery endothelial dysfunction in response to a Western diet. (A) The area under the curve (AUC) of all voltages of the voltage-dependent erectile response was attenuated 8 weeks into the Western diet. (B) ACh-stimulated vasodilation of the coronary artery was attenuated 12 weeks following initiation of the Western diet (elevated EC_{50}). * $P < 0.05$ vs. Control group, ** $P < 0.01$. Reported are means ± SEM for 5-7 animals in each group.

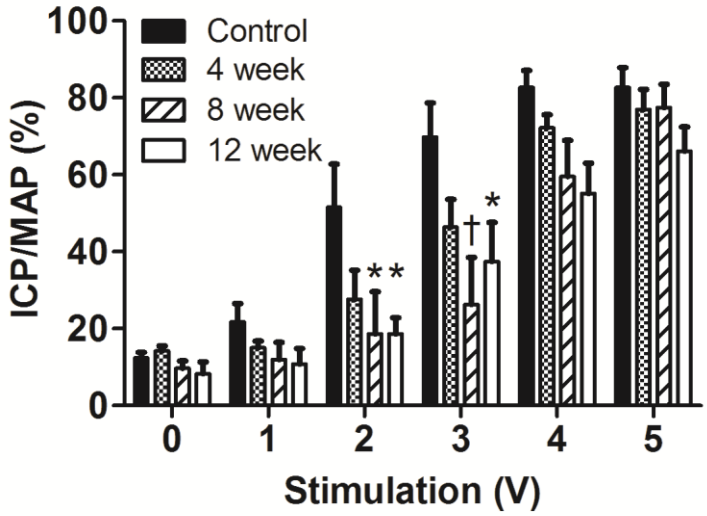

Figure 2 Voltage-dependent erectile response following control diet or Western diet for 4, 8, or 12 weeks. ICP/MAP was reduced at 2 and 3 volts in the 8-week (hatched bars) and 12-week (open bars) Western diet fed rats compared to control diet fed rats (black bars). Reported are means ± SEM for 5-7 animals in each group; * $P < 0.05$, † $P < 0.01$ vs. Control.

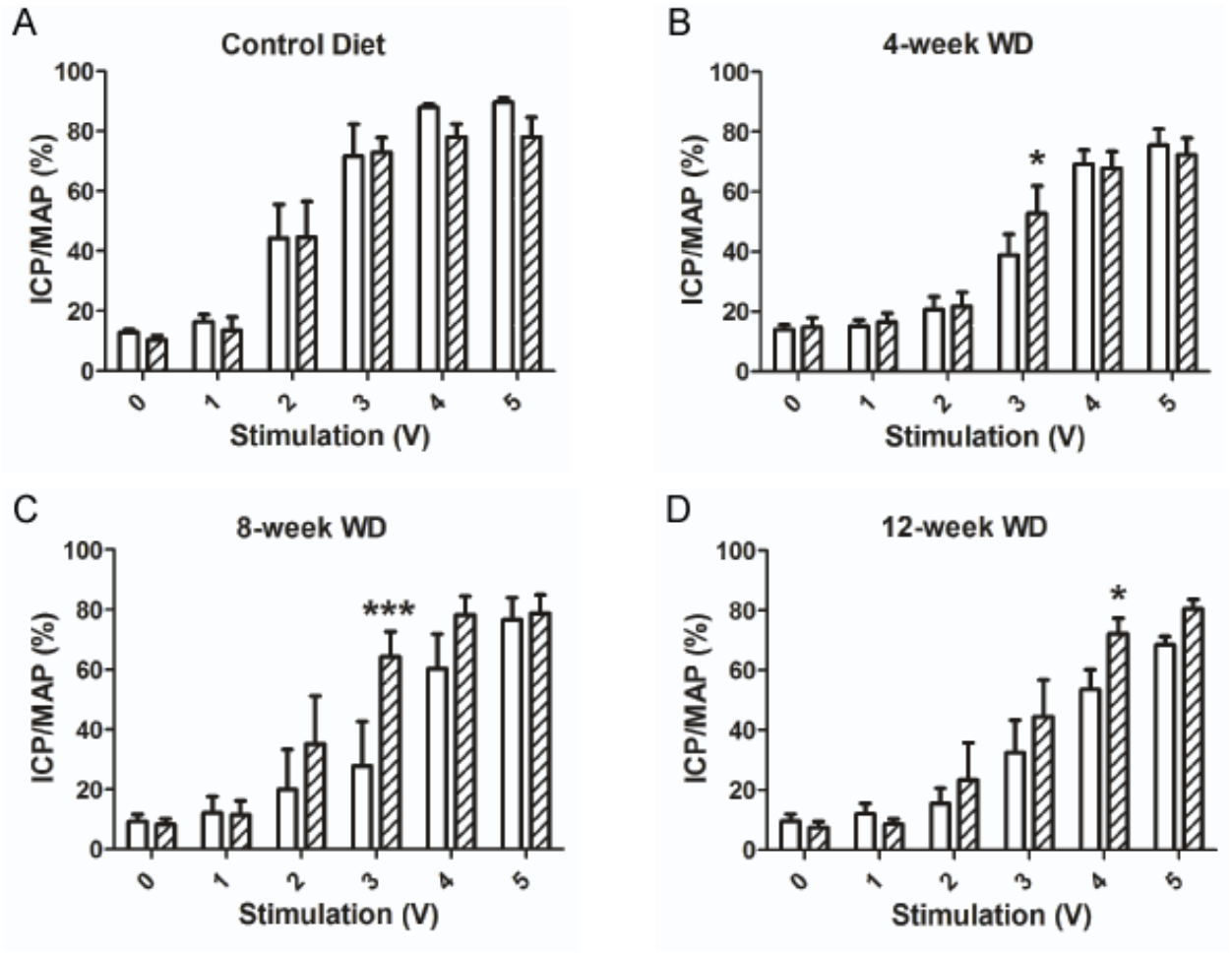

Figure 3 Effect of sepiapterin on voltage-dependent erectile response. Following the untreated voltage series' (open bars), 10 μM sepiapterin was injected intracavernosally and a voltage series was applied 30-minutes post-injection (hatched bars). No treatment effects were observed in rats fed the control diet (A), while rats fed the Western diet for 4 weeks (B), 8 weeks (C), and 12 weeks (D) demonstrated an enhanced erectile response with sepiapterin treatment. * $P < 0.05$, *** $P < 0.001$. Reported are means ± SEM for 4-6 animals in each group.

Coronary Artery Endothelium-Dependent and -Independent Relaxation

Coronary artery endothelial function was assessed from the concentration-response to the endothelium-dependent agonist acetylcholine. The effective concentration producing 50% of a maximal relaxation response (EC_{50}) following pre-constriction was used to assess sensitivity to acetylcholine, and thus the ability of the endothelium to produce nitric oxide. The EC_{50} of the acetylcholine response was significantly elevated in 12-week Western diet fed rats compared to all other groups (Figure 1B). The ACh-induced relaxation curves are plotted in Figure 4A, where the relaxation profile was significantly attenuated at a single concentration for the 8-week WD rats and at three concentrations for the 12-week WD rats when compared to the relaxation profiles of control diet rats. There were no significant differences in the Hill-slope for the ACh response (Control: 1.01 ± 0.23; 4-wk: 0.96 ± 0.16; 8-wk: 1.01 ± 0.21; 12-wk: 0.64 ± 0.09; P = 0.522). Coronary artery endothelium-independent relaxation was assessed from the concentration-response profile to the NO donor sodium nitroprusside. There were no significant differences between groups for the relaxation response to SNP (Figure 4B), indicating no diet-induced differences in vessel response to NO. There were no significant differences for the EC_{50} (Control: 25.1 ± 0.23 nM; 4-wk: 21.7 ± 6.8 nM; 8-wk: 19.9 ± 8.6 nM; 12-wk: 14.5 ± 6.0 nM; P = 0.821) or the Hill-slope (Control: 1.48 ± 0.18; 4-wk: 1.45 ± 0.24; 8-wk: 1.47 ± 0.24; 12-wk: 1.54 ± 0.16; P = 0.994) values for the SNP response.

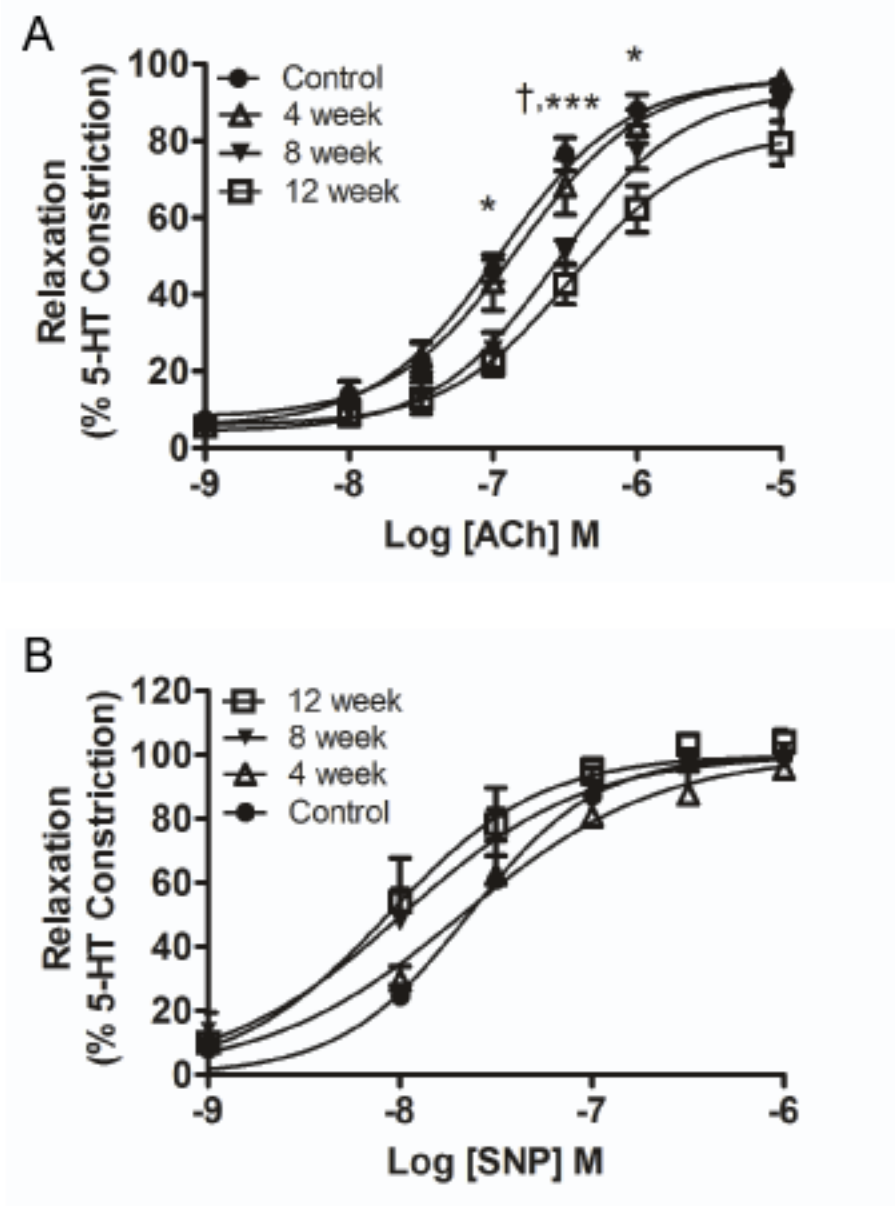

Figure 4 Mean concentration-response of left-anterior descending coronary artery segments following 3.0 µM 5-HT pre-constriction to (A) acetylcholine stimulation (0.001 – 10.0 µM) or (B) sodium nitroprusside stimulation (0.001 – 1.0 µM) in rats exposed to the control diet or Western diet for 4, 8, or 12 weeks. Reported are means ± SEM for 5-7 animals in each group. † $P < 0.05$ 8-week vs. Control, * $P < 0.05$ 12-week vs. Control, *** $P < 0.001$ 12-week vs. Control.

Animal Study: Aim 2

Metabolic Parameters

The body weight from the CD-Ex rats was significantly lower than WD-Sed, however WD-Sed rats had significantly more fat mass than any other group while lean mass was not different among any group (Table 7). Despite increased glucose in the WD-Sed rats, no differences in insulin, HOMA-IR or HOMA-Beta (Table 7) were observed between groups. WD-Ex rats had lower total cholesterol levels than CD-Sed rats, while no differences existed for HDL-cholesterol, LDL-cholesterol, or triglyceride levels between groups (Table 7).

Table 7 Aim 2 Metabolic Parameters

	CD - Sed	CD - Ex	WD - Sed	WD - Ex	ANOVA P-value
Body Weight (g)	458 ± 8.0	424 ± 12.5	499 ± 11.1†	456 ± 17.2	0.003
Fat Mass (g)	52.0 ± 5.97	34.8 ± 7.48	76.9 ± 4.93*,†,‡	44.3 ± 8.48	0.001
Lean Mass (g)	341 ± 6.1	328 ± 7.8	356 ± 8.3	349 ± 10.3	0.154
Body Fat %	13.1 ± 1.30	9.3 ± 1.82	17.7 ± 0.98†,‡	10.9 ± 1.52	0.001
Glucose (mg/dl)	107 ± 3.5	99 ± 1.6	121 ± 3.5*,†	109 ± 3.7	<0.001
Insulin (pM)	125 ± 43.5	142 ± 28.1	174 ± 24.3	116 ± 23.4	0.482
HOMA-IR	4.94 ± 1.84	4.87 ± 0.95	7.34 ± 1.04	4.51 ± 1.09	0.250
HOMA-Beta	123 ± 36.5	197 ± 41.8	150 ± 21.7	123 ± 17.5	0.321
Cholesterol (mg/dl)	39.0 ± 4.53‡	34.0 ± 2.59	30.2 ± 1.91	27.1 ± 2.14	0.029
HDL-C (mg/dl)	25.9 ± 2.36	22.9 ± 2.24	24.6 ± 1.67	21.8 ± 1.80	0.542
LDL-C (mg/dl)	8.6 ± 1.44	9.8 ± 0.66	7.7 ± 0.42	8.0 ± 0.50	0.129
Triglycerides (mg/dl)	58.0 ± 9.8	54.8 ± 12.2	45.3 ± 10.0	27.3 ± 6.1	0.128

Values are means ± SEM, N = 5-11 animals per group. * Significant vs. CD-Sed, † Significant vs. CD-Ex, ‡ Significant vs. WD-Ex. CD, Control diet; WD, Western diet; Sed, Sedentary; Ex, Exercise.

Voltage-Dependent Erectile Response

The ICP/MAP in response to the intermediate voltages (2, 3, and 4V) was significantly attenuated by the Western diet, and was preserved by exercise training within the Western diet (Figure 5). However, the ICP/MAP at 2 and 3V was significantly attenuated by exercise training within the control diet (Figure 5). Intracavernosal treatment with apocynin (Figure 6) or sepiapterin (Figure 7) had no effect on the voltage-dependent erectile response of the CD-Sed, CD-Ex, or WD-Ex rats, but significantly augmented the erectile response of WD-Sed rats.

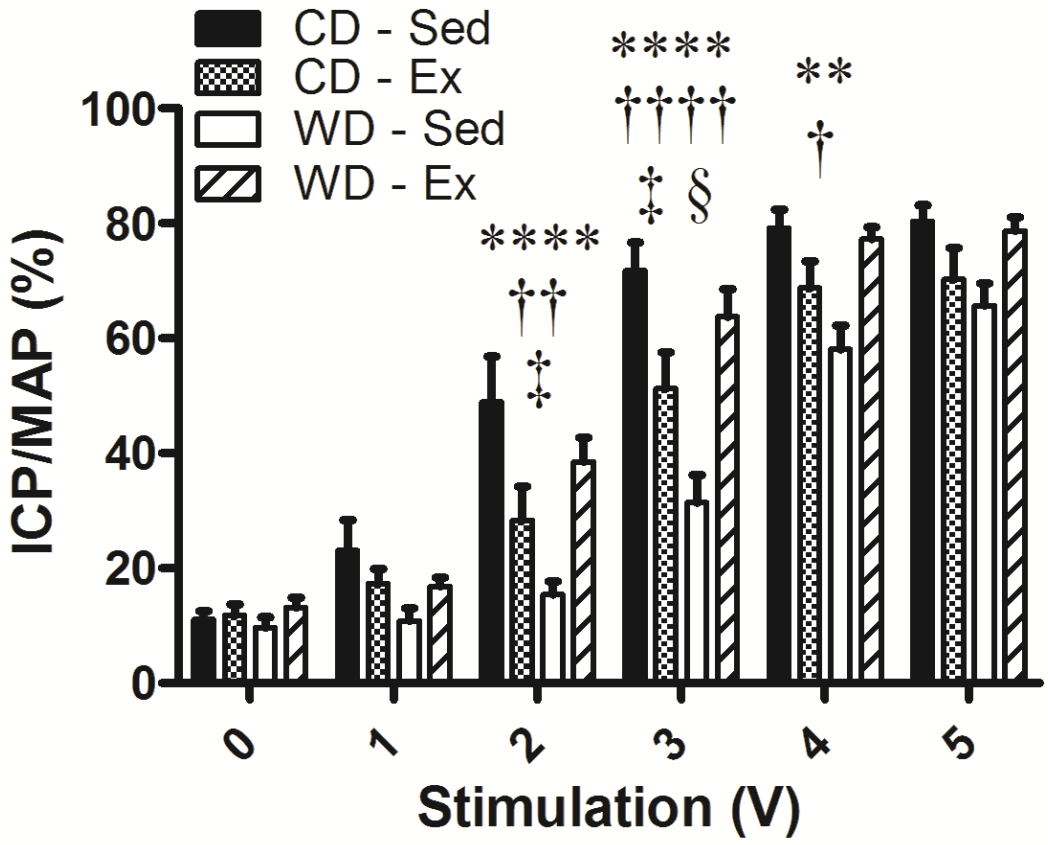

Figure 5 Voltage-dependent erectile response. ICP/MAP was attenuated by the Western diet, which was prevented by exercise training. ICP/MAP was attenuated by exercise training within the control diet. Reported are means ± SEM for 8-11 animals in each group; **** P < 0.0001 CD-Sed vs. WD-Sed, ** P < 0.01 CD-Sed vs. WD-Sed, †††† P < 0.0001 WD-Ex vs. WD-Sed, †† P < 0.01 WD-Ex vs. WD-Sed, † P < 0.05 WD-Ex vs. WD-Sed, ‡ P < 0.05 CD-Sed vs. CD-Ex. § P < 0.05 CD-Ex vs. WD-Sed. CD, control diet; WD, Western diet; Ex, exercise; Sed, sedentary.

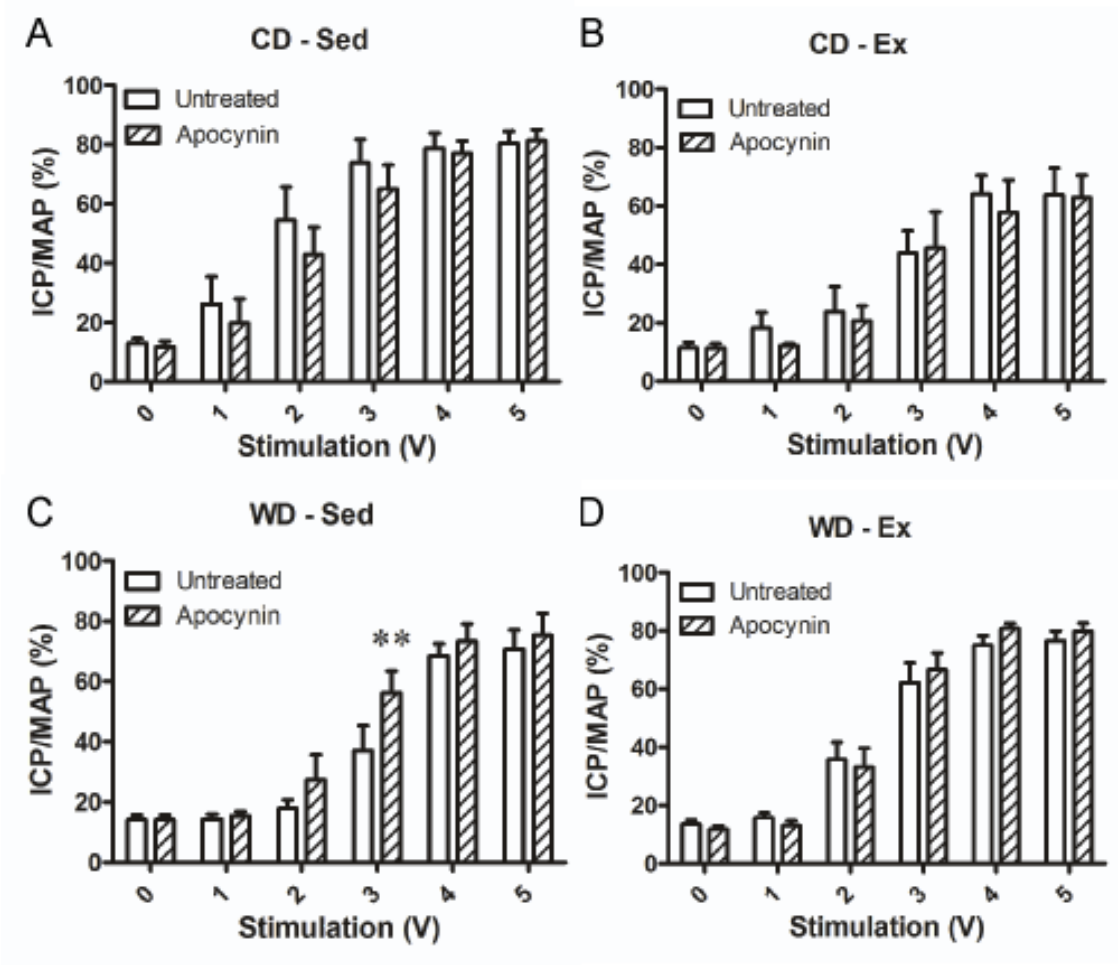

Figure 6 Effect of acute apocynin administration on voltage-dependent erectile response. Following the untreated voltage series' (open bars), 1 mM apocynin was injected intracavernosally and a voltage series was applied 20-minutes post-injection (hatched bars). No treatment effects were observed in control diet-sedentary (CD-Sed) (A), control diet-exercise (CD-Ex) (B), or Western diet-exercise (WD-Ex) (D), while apocynin enhanced the erectile response in Western diet-sedentary (WD-Sed) rats (C). Reported are means ± SEM for 4-5 animals in each group ** $P < 0.01$ vs. untreated.

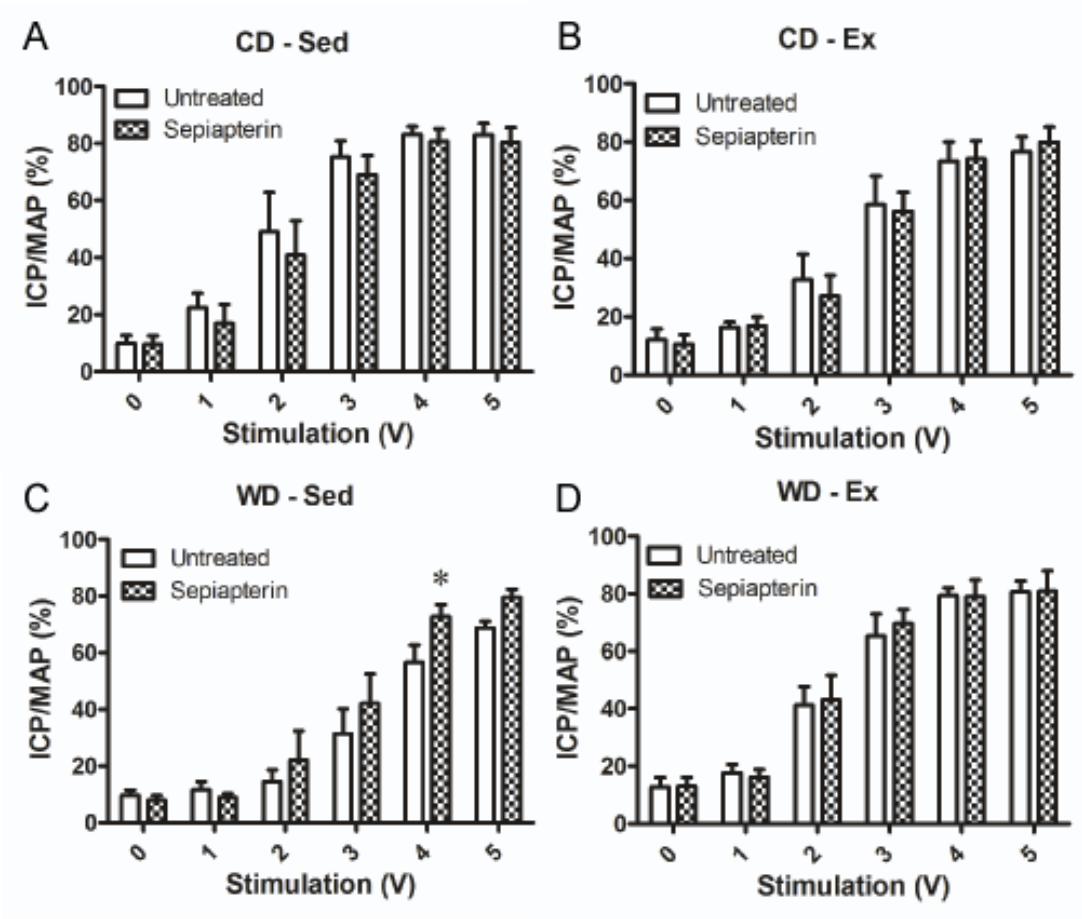

Figure 7 Effect of acute sepiapterin administration on voltage-dependent erectile response. Following the untreated voltage series' (open bars), 10 μM sepiapterin was injected intracavernosally and a voltage series was applied 30-minutes post-injection (checkered bars). No treatment effects were observed in control diet-sedentary (CD-Sed) (A), control diet-exercise (CD-Ex) (B), or Western diet-exercise (WD-Ex) (D), while sepiapterin enhanced the erectile response in Western diet-sedentary (WD-Sed) rats (C). Reported are means ± SEM for 4-6 animals in each group * $P < 0.05$ vs. untreated.

Coronary Artery Endothelium-Dependent and -Independent Relaxation

The ACh-stimulated relaxation curves are presented in Figure 8A. The relaxation profile was significantly attenuated by the Western diet at two ACh concentrations. Similarly, exercise augmented the relaxation profile within the Western diet at two ACh concentrations. The coronary artery endothelium-independent relaxation was assessed from the concentration-response profile to the NO donor sodium nitroprusside. There were no significant differences between groups for the relaxation response to SNP (Figure 8B), indicating no diet or exercise induced differences in vessel response to NO. Thus, the impaired ACh response in WD-Sed rats is likely a dysfunction of the endothelium via impaired NO production as the smooth muscle retains a normal relaxation response to a NO donor and activator of the cGMP mediated relaxation pathway. Treatment of the vessel segments with apocynin augmented ACh-stimulated vasorelaxation profile in WD-Sed, but had no significant effect on Con-Sed, Con-Ex, or WD-Ex coronary artery segments (Figure 9). Treatment with sepiapterin attenuated the ACh-stimulated vasorelaxation profile in Con-Sed, but had no significant effect on Con-Ex, WD-Sed, or WD-Ex coronary artery segments (Figure 10).

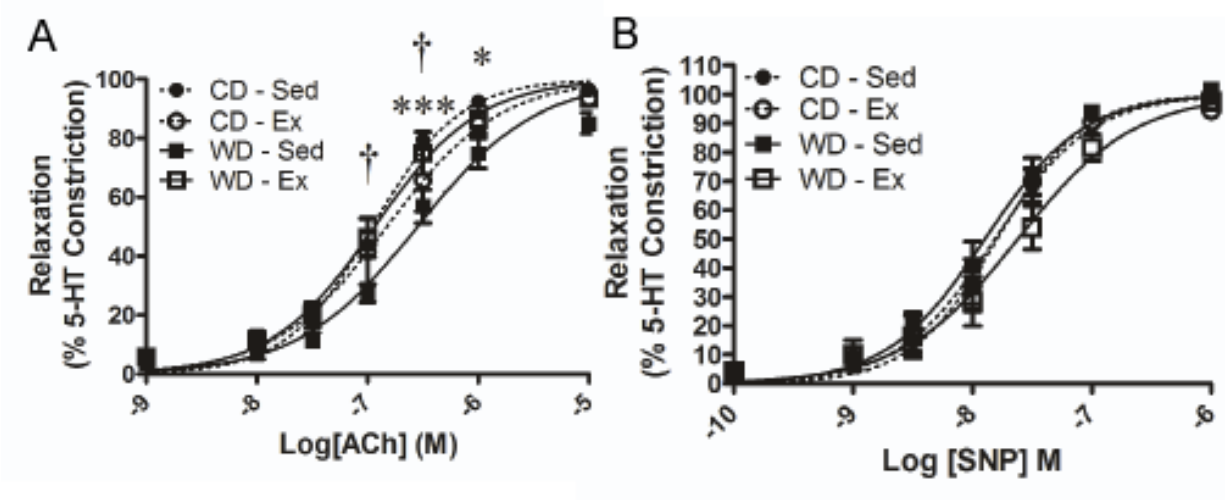

Figure 8 Mean concentration-response of coronary artery segments following 3.0 µM 5-HT pre-constriction to (A) acetylcholine (ACh) stimulation (0.001 – 10.0 µM) or (B) sodium nitroprusside (SNP) stimulation (0.0001 – 1.0 µM). Mean ACh-response EC_{50} values were: 0.111 µM (CD-Sed), 0.150 µM (CD-Ex), 0.283 µM (WD-Sed), 0.116 µM (WD-Ex). Mean SNP-response EC_{50} values are: 16.1 nM (CD-Sed), 15.5 nM (CD-Ex), 12.7 nM (WD-Sed), 24.1 nM (WD-Ex). Reported are means ± SEM for 6-11 animals in each group. *** $P < 0.001$ CD-Sed vs. WD-Sed,* $P < 0.05$ CD-Sed vs. WD-Sed, † $P < 0.05$ WD-Ex vs. WD-Sed. CD, control diet; WD, Western diet; Ex, exercise; Sed, sedentary.

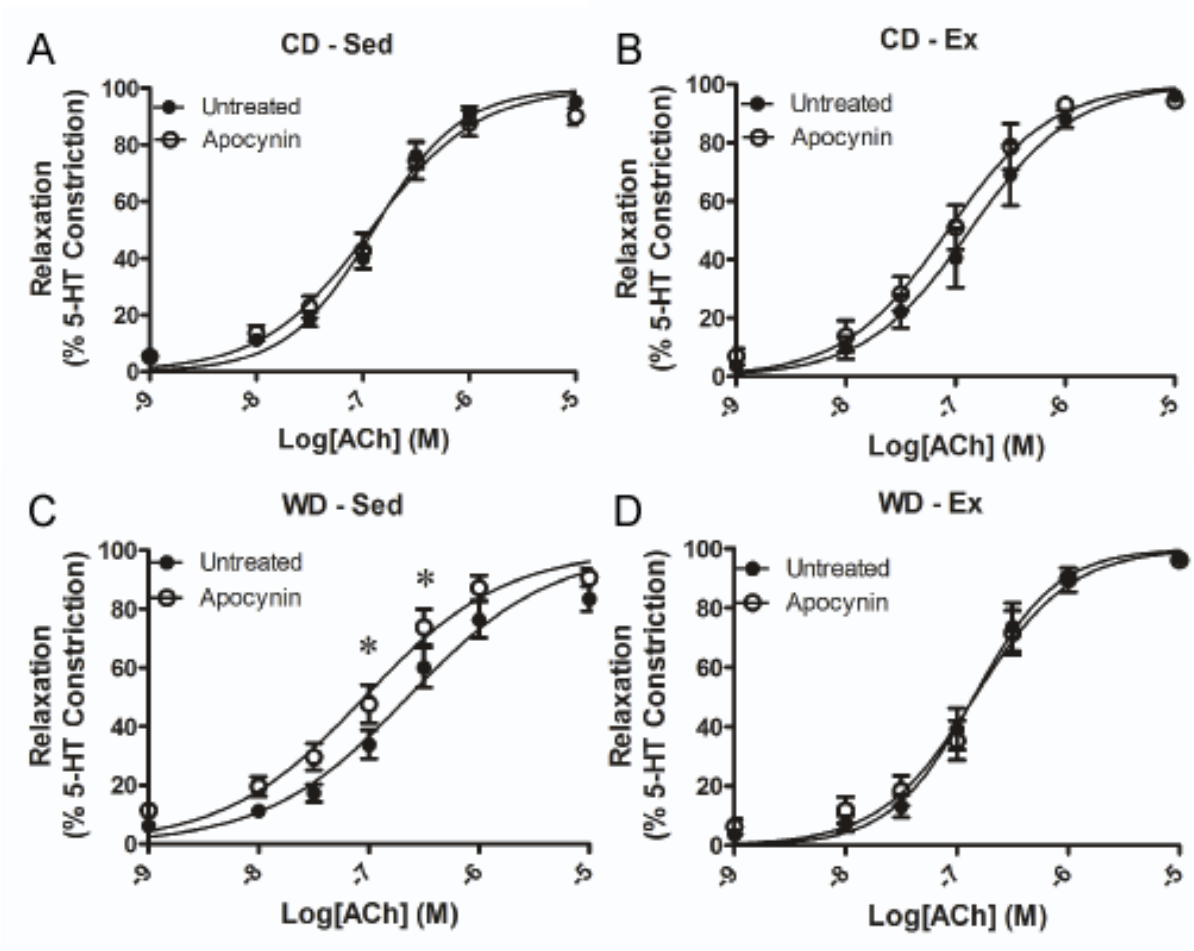

Figure 9 Effect of apocynin on acetylcholine (ACh)-stimulated vasorelaxation of coronary artery segments. Vasorelaxation was examined in the absence (closed circles) and presence (open circles) of 300 µM apocynin. No treatment effects were observed in control diet-sedentary (CD-Sed) (A), control diet-exercise (CD-Ex) (B), or Western diet-exercise (WD-Ex) (D), while apocynin enhanced ACh-stimulated vasorelaxation in Western diet-sedentary (WD-Sed) rats (C). Mean response EC_{50} values are: 0.126 vs. 0.118 µM (CD-Sed), 0.135 vs. 0.085 µM (CD-Ex), 0.226 vs. 0.092 µM (WD-Sed), 0.142 vs. 0.145 µM (WD-Ex), untreated vs. apocynin, respectively. Reported are means ± SEM for 6-10 animals in each group * $P < 0.05$ vs. untreated.

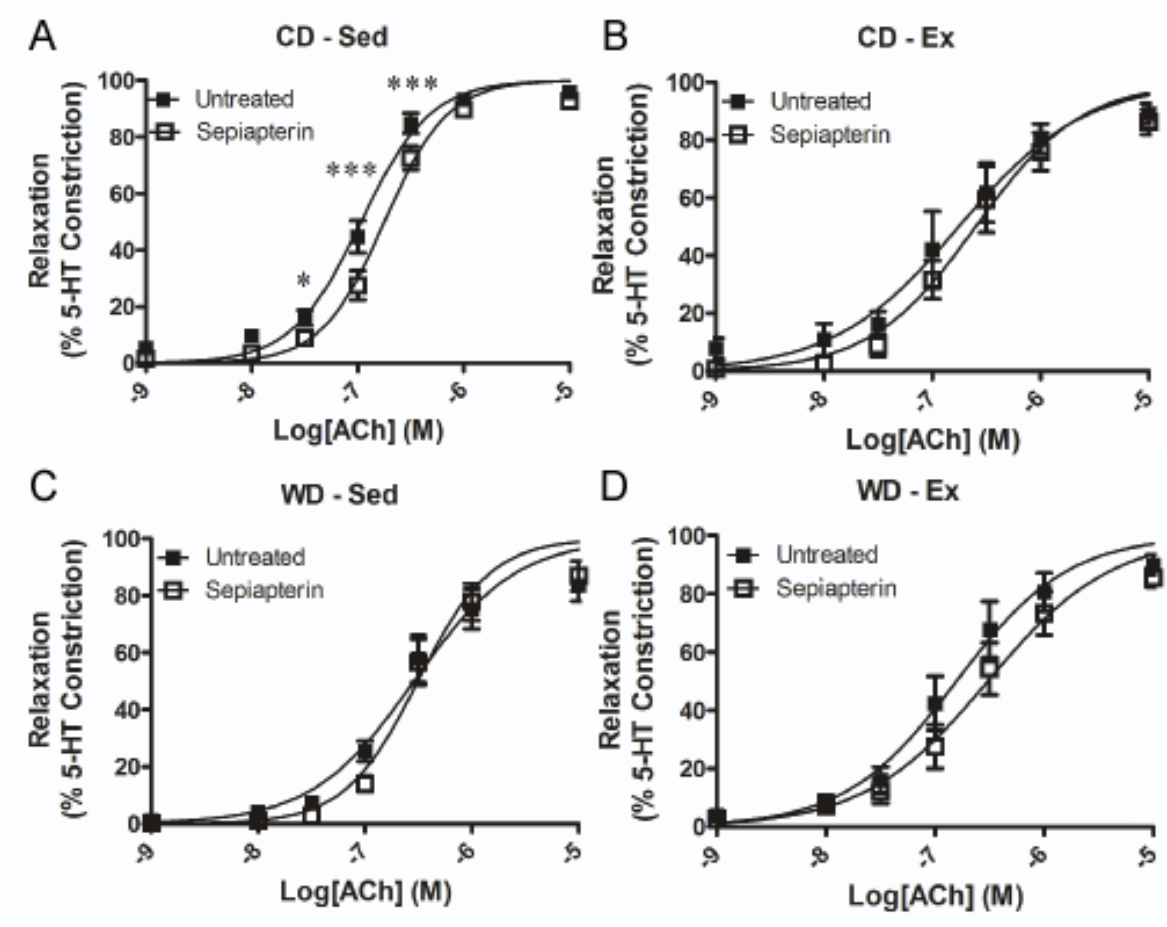

Figure 10 Effect of sepiapterin on acetylcholine (ACh)-stimulated vasorelaxation of coronary artery segments. Vasorelaxation was examined in the absence (closed squares) and presence (open squares) of 10 µM sepiapterin. Sepiapterin treatment attenuated ACh-stimulated vasorelaxation in control diet-sedentary rats (A), while no treatment effects were observed in control diet-exercise (B), Western diet-sedentary (WD-Sed) (C), or Western diet-exercise (WD-Ex) (D) rats. Mean response EC_{50} values are: 0.107 vs. 0.179 µM (CD-Sed), 0.176 vs. 0.252 µM (CD-Ex), 0.288 vs. 0.313 µM (WD-Sed), 0.157 vs. 0.305 µM (WD-Ex), untreated vs. sepiapterin, respectively. Reported are means ± SEM for 6-10 animals in each group * $P < 0.05$ vs. untreated, *** $P < 0.001$ vs. untreated.

Human Study: Aim 3

Pilot Data

Pilot data was collected from lean (BMI < 25 kg/m^2) and obese (BMI > 30 kg/m^2) subjects. Measurement of *in vivo* ROS production is presented in Figure 11. There were no apparent differences in ROS production between lean subjects and those with a BMI ranging from 30.5-31.5 kg/m^2 (P = 0.894). However, there was an apparent elevation in ROS production in subjects with a BMI ranging from 33-38 kg/m^2 compared to lean subjects and subjects with a BMI from 30.5-31.5 kg/m^2 (Figure 11).

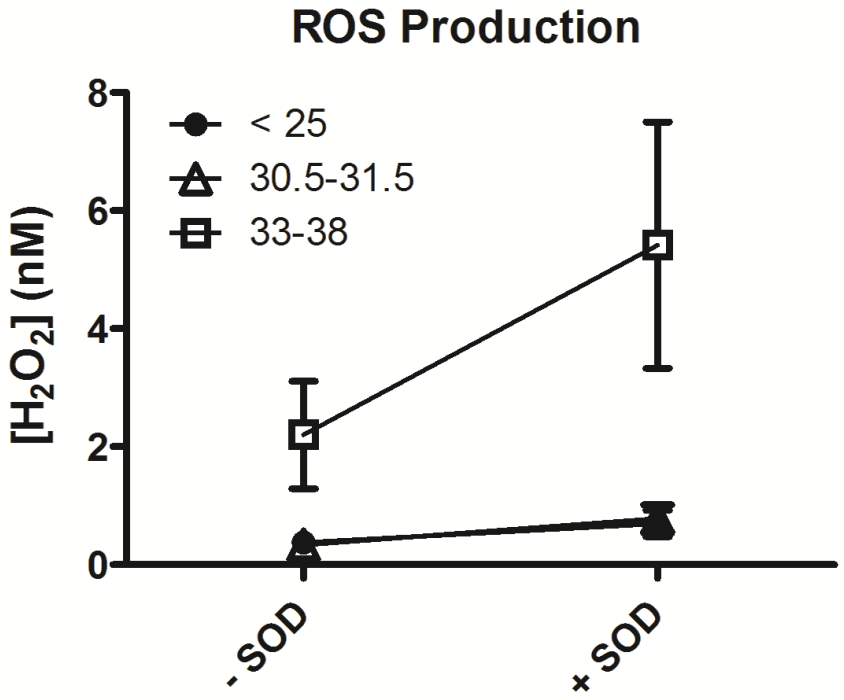

Figure 11 Pilot data of *in vivo* ROS production in lean subjects (BMI < 25, n = 5), subjects with a BMI of 30.5-31.5 (n = 3), and subjects with a BMI of 33-38 (n = 4). ROS was measured via Amplex Ultrared fluorescence in the absence of SOD (-SOD), indicative of endogenous H_2O_2 that crosses the microdialysis membrane, and in the presence of SOD (+SOD), indicative of endogenous H_2O_2 that crosses the microdialysis membrane and superoxide that crosses the microdialysis membrane and is converted to H_2O_2 within the membrane. Values are mean ± SEM. SOD, superoxide dismutase; H_2O_2, hydrogen peroxide.

Subject Characteristics and Metabolic Parameters

Subjects in tertile 2 were older than tertile 1 ($P < 0.05$), while there was no significant age difference between tertile 1 and tertile 3 (Table 8). There was a progressive increase in body fat percentage across the BMI tertiles ($P < 0.001$). Despite increased adiposity, there were no significant differences between tertiles in fasting blood glucose or insulin concentrations, corresponding to no significant differences in HOMA-1 (Table 8). Additionally, there were no significant differences between tertiles in fasting triglycerides, total-cholesterol, LDL-cholesterol, or HDL-cholesterol (Table 8). There were no significant differences between tertiles in absolute VO_{2peak}, but a significant decrease in VO_{2peak} relative to body mass across the tertiles ($P < 0.001$, Table 8).

Table 8 Subject characteristics and metabolic parameters of each BMI tertile

	1	2	3	P-Value
Age (years)	22.4 ± 0.56	29.7 ± 2.87*	24.1 ± 1.23	0.038
Height (cm)	174 ± 3.15	176 ± 3.03	167 ± 3.25	0.141
Weight (kg)	66.7 ± 4.76	95.6 ± 4.22*	105.9 ± 5.70*	<0.001
BMI (kg/m^2)	21.9 ± 1.00	30.6 ± 0.53*	37.6 ± 1.01*,†	<0.001
Body Fat %	22.6 ± 3.07	36.0 ± 1.90*	47.2 ± 0.91*,†	<0.001
Glucose (mg/dl)	84.7 ± 3.41	92.6 ± 2.23	90.4 ± 2.67	0.147
Insulin (μIU/ml)	6.62 ± 1.34	16.3 ± 5.20	14.4 ± 2.67	0.220
HOMA-IR	1.40 ± 0.30	3.84 ± 1.37	3.31 ± 0.70	0.248
Triglycerides (mg/dl)	93.0 ± 8.1	113.4 ± 22.2	82.6 ± 13.7	0.428
Total-Cholesterol (mg/dl)	163.8 ± 15.4	159.8 ± 8.8	160.6 ± 13.2	0.972
HDL-Cholesterol (mg/dl)	54.3 ± 7.74	41.9 ± 3.53	48.0 ± 5.37	0.297
LDL-Cholesterol (mg/dl)	90.9 ± 10.4	95.3 ± 9.8	96.1 ± 13.3	0.946
$VO2_{peak}$ (l/min)	2.62 ± 0.26	2.93 ± 0.30	2.42 ± 0.17	0.361
$VO2_{peak}$ ($ml \cdot kg^{-1} \cdot min^{-1}$)	38.9 ± 2.02	30.1 ± 2.41*	22.7 ± 1.16*,†	<0.001

Values are mean ± SEM. * $P < 0.05$ vs. tertile 1, † $P < 0.05$ vs. tertile 2.

In Vivo ROS Production

In vivo ROS production was measured with and without SOD added to the perfusate. Amplex ultrared fluorescence without SOD in the perfusate is indicative of H_2O_2 produced endogenously that crosses over the microdialysis membrane (Figure 12a). With SOD added to the perfusate, Amplex ultrared fluorescence is indicative of H_2O_2 produced endogenously that crosses over the microdialysis membrane in addition to H_2O_2 produced inside the microdialysis probe by SOD from superoxide that crosses over the microdialysis membrane (Figure 12b). Endogenous H_2O_2 production was not significantly different across tertiles ($P = 0.067$). However, there was a significant tertile*apocynin interaction effect ($P = 0.049$), indicating a greater effect of NADPH oxidase inhibition on H_2O_2 production in tertile 3. Post hoc analysis indicates that H_2O_2 production was significantly reduced when apocynin was added to the perfusate in tertile 3 ($P < 0.001$). With SOD added to the perfusate, there was significant effect of BMI on ROS production ($P = 0.030$). Post hoc analysis indicates that ROS production was significantly ($P < 0.05$) increased in tertile 3 compared to both tertile 1 and tertile 2 (Figure 12b). There was a significant tertile*apocynin interaction effect ($P = 0.023$), indicating a greater effect of NADPH oxidase inhibition on combined H_2O_2 and superoxide production in tertile 3. Post hoc analysis indicates that ROS production is significantly reduced when apocynin is added to the perfusate in tertile 3 (Figure 12b; $P < 0.01$).

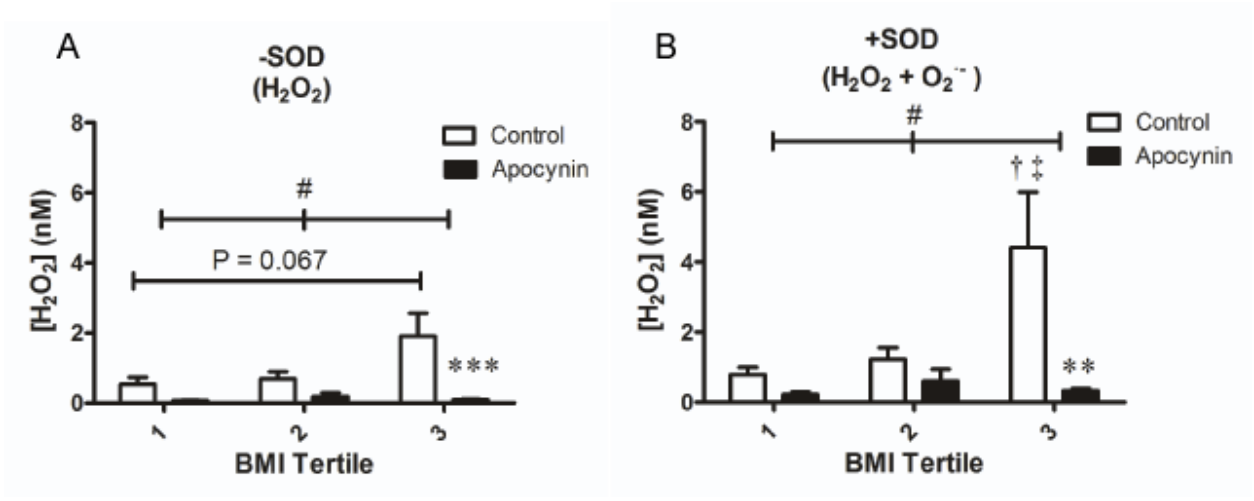

Figure 12 In vivo reactive oxygen species (ROS) production was measured in the absence (control, open bars) and presence of apocynin (closed bars), an NADPH oxidase inhibitor. In vivo ROS production was measured without superoxide dismutase (SOD) added to the perfusate, which is indicative of H_2O_2 produced endogenously that crosses over the microdialysis membrane (A). ROS detected with SOD added to the perfusate is indicative of H_2O_2 produced endogenously that crosses over the microdialysis membrane in addition to H_2O_2 produced inside the microdialysis probe by SOD from superoxide that crosses over the microdialysis membrane (B). Endogenous H_2O_2 production was not significantly different across tertiles ($P = 0.067$), while combined H_2O_2 and superoxide production were significantly elevated in tertile 3. Addition of apocynin to the perfusate significantly attenuated ROS production in tertile 3. Values are mean ± SEM for n = 7, 8, 7 subjects in tertiles 1,2, and 3, respectively. † $P < 0.05$ vs. tertile 1. ‡ $P < 0.05$ vs. tertile 2. # Significant ($P < 0.05$) tertile*apocynin interaction effect. ** Significant treatment effect of apocynin ($P < 0.01$). *** Significant treatment effect of apocynin ($P < 0.001$).

Microvascular Blood Flow Responses

Microvascular endothelial function was assessed by measurement of ACh-stimulated blood flow. The impact of NADPH oxidase on microvascular endothelial function was assessed by co-perfusion of ACh and apocynin (Figure 13). ACh-stimulated blood flow was significantly depressed in tertile 3 ($P = 0.011$). Post hoc analysis indicates that ACh-stimulated blood flow was significantly decreased compared to tertile 1 ($P < 0.05$) and tertile 2 ($P < 0.05$). There were no significant differences in ACh-stimulated blood flow between tertile 1 and tertile 2. There was a significant tertile*apocynin effect on ACh-stimulated blood flow ($P = 0.048$), indicating an increase in ACh-stimulated blood flow with apocynin that is specific to tertile 3 (Figure 13). Microvascular endothelial function was further probed by perfusion of SNP, a direct NO donor and activator of the cGMP pathway (Figure 14). There were no significant differences in SNP-stimulated blood flow across tertiles ($P = 0.489$). There were also no significant differences across tertiles in basal, non-stimulated blood flow ($P = 0.201$, Figure 14).

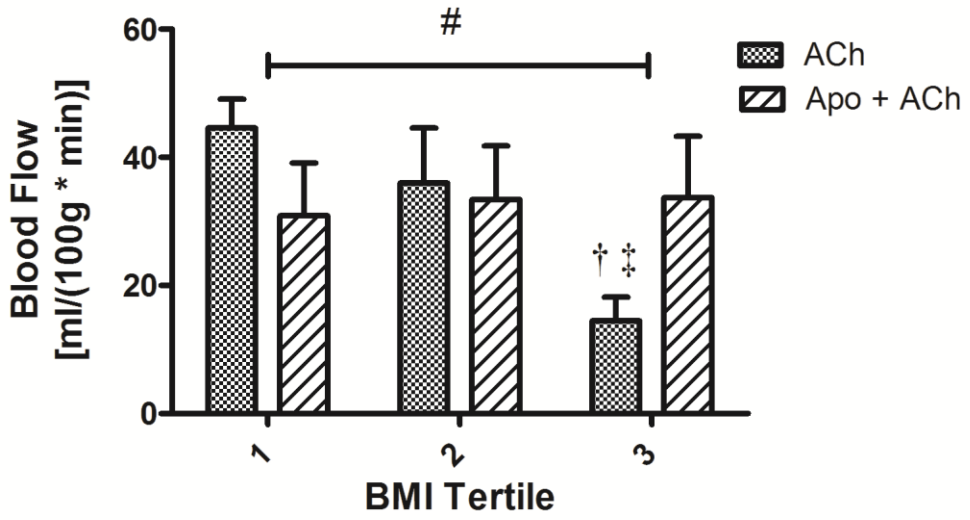

Figure 13 The effect of BMI and apocynin, an NADPH oxidase inhibitor, on microvascular endothelial function assessed by acetylcholine (ACh)-stimulated blood flow. ACh-stimulated blood flow was depressed in tertile 3. Apocynin augmented ACh-stimulated blood flow only in tertile 3. Values are mean ± SEM for n = 7, 8, 7 subjects in tertiles 1,2, and 3, respectively. † $P < 0.05$ vs. tertile 1. ‡ $P < 0.05$ vs. tertile 2. # Significant ($P < 0.05$) tertile*apocynin interaction effect.

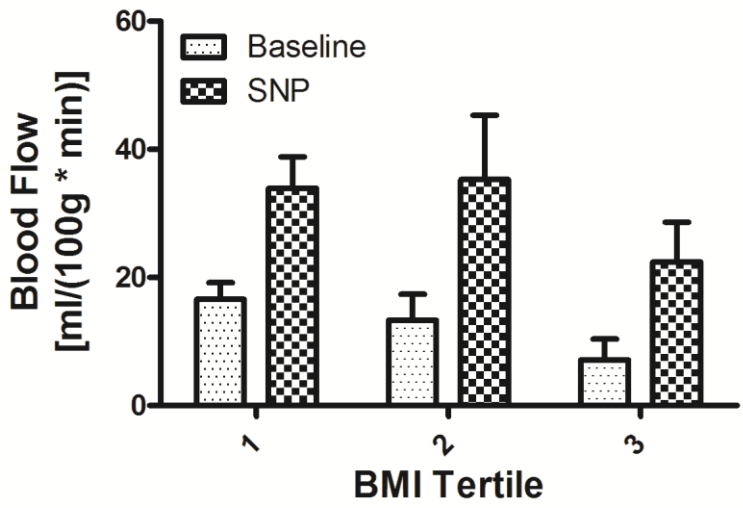

Figure 14 Effect of BMI on basal blood flow and endothelium independent vasodilation. There were no differences in basal, non-stimulated blood flow across BMI tertiles. Stimulation of blood flow independent of the endothelium was assessed by perfusion of sodium nitroprusside (SNP). There were no differences in SNP-stimulated blood flow across BMI tertiles. Values are mean ± SEM for n = 7, 8, 7 subjects in tertiles 1,2, and 3, respectively.

Effects of Exercise Training Intervention on Metabolic Parameters

Due to a subject drop-out rate that was higher than anticipated and no significant pre-training differences in ROS production or microvascular endothelial function between tertiles 1 and 2, the exercise training responses for tertiles 1 and 2 were combined to compare to tertile 3. There were no significant effects of the aerobic interval training intervention on body weight, glucose, insulin, blood lipid profile, or peak aerobic capacity (Table 9).

Table 9 Effect of exercise intervention on metabolic parameters

	Tertiles 1 & 2			Tertile 3		
	Pre-training	Post-training	P	Pre-training	Post-training	P
BMI (kg/m^2)	25.6 ± 2.85	25.5 ± 3.02	0.953	36.0 ± 1.17	36.0 ± 1.16	0.945
Body Fat %	28.2 ± 5.83	27.8 ± 5.97	0.454	46.6 ± 2.06	47.0 ± 1.31	0.692
Glucose (mg/dl)	87.6 ± 5.12	84.4 ± 4.41	0.378	90.0 ± 4.10	97.0 ± 4.08	0.063
Insulin (μIU/ml)	6.42 ± 1.69	7.44 ± 2.60	0.776	11.8 ± 3.49	17.4 ± 3.04	0.090
HOMA-IR	1.42 ± 0.42	1.62 ± 0.59	0.808	2.71 ± 0.92	4.24 ± 0.89	0.068
Triglycerides (mg/dl)	68.6 ± 4.96	58.8 ± 7.22	0.359	91.8 ± 17.3	113 ± 25.9	0.185
Total-Cholesterol (mg/dl)	156 ± 15.1	156 ± 17.8	0.995	165 ± 19.9	163 ± 14.2	0.844
HDL-Cholesterol (mg/dl)	50.4 ± 9.57	50.6 ± 6.47	0.980	43.3 ± 3.17	37.8 ± 2.29	0.163
LDL-Cholesterol (mg/dl)	92.3 ± 14.0	93.8 ± 20.7	0.962	104 ± 14.4	103 ± 8.24	0.920
$VO2_{peak}$ (l/min)	2.41 ± 0.28	2.76 ± 0.33	0.064	2.71 ± 0.33	3.06 ± 0.22	0.240
$VO2_{peak}$ ($ml \bullet kg^{-1} \bullet min^{-1}$)	31.3 ± 3.93	35.3 ± 3.13	0.163	25.2 ± 0.58	29.1 ± 2.78	0.225

Values are mean ± SEM for 5 subjects in tertiles 1 & 2, and 4 subjects in tertile 3.

Effects of Exercise Training Intervention on in vivo ROS Production

There were no significant training induced changes in H_2O_2 production (P = 0.09; effect size = 0.837; Figure 15a) or H_2O_2 that is not blocked by apocynin (P = 0.852; effect size 0.145; Figure 15a) in tertiles 1 and 2. There was no significant training*apocynin interaction on H_2O_2 production in tertiles 1 and 2 (P = 0.100; Figure 15a). Similarly, there were no significant training induced changes in combined H_2O_2 and superoxide production (P = 0.065; effect size = 0.714; Figure 15c) or combined H_2O_2 and superoxide production that is not blocked by apocynin (P = 0.910; effect size = 0.090; Figure 15c) in tertiles 1 and 2. There was no significant training*apocynin interaction on combined H_2O_2 and superoxide production in tertiles 1 and 2 (P = 0.261; Figure 15c). There was no significant training induced change in H_2O_2 production (P = 0.416; effect size = 0.702; Figure 15b) in tertile 3, however, there was a significant training induced increase in H_2O_2 production that is not blocked by apocynin (P = 0.035; effect size = 1.393; Figure 15b). There was no significant training*apocynin interaction on H_2O_2 production (P = 0.315; Figure 15c). Similarly, there was no significant training induced change in combined H_2O_2 and superoxide production (P = 0.797; effect size = 0.268; Figure 15d) in tertile 3, however, there was a significant increase in combined H_2O_2 and superoxide production (P = 0.015, effect size = 1.383, Figure 15d). There was no significant training*apocynin interaction on combined H_2O_2 and superoxide production in tertile 3 (P = 0.670; Figure 15d).

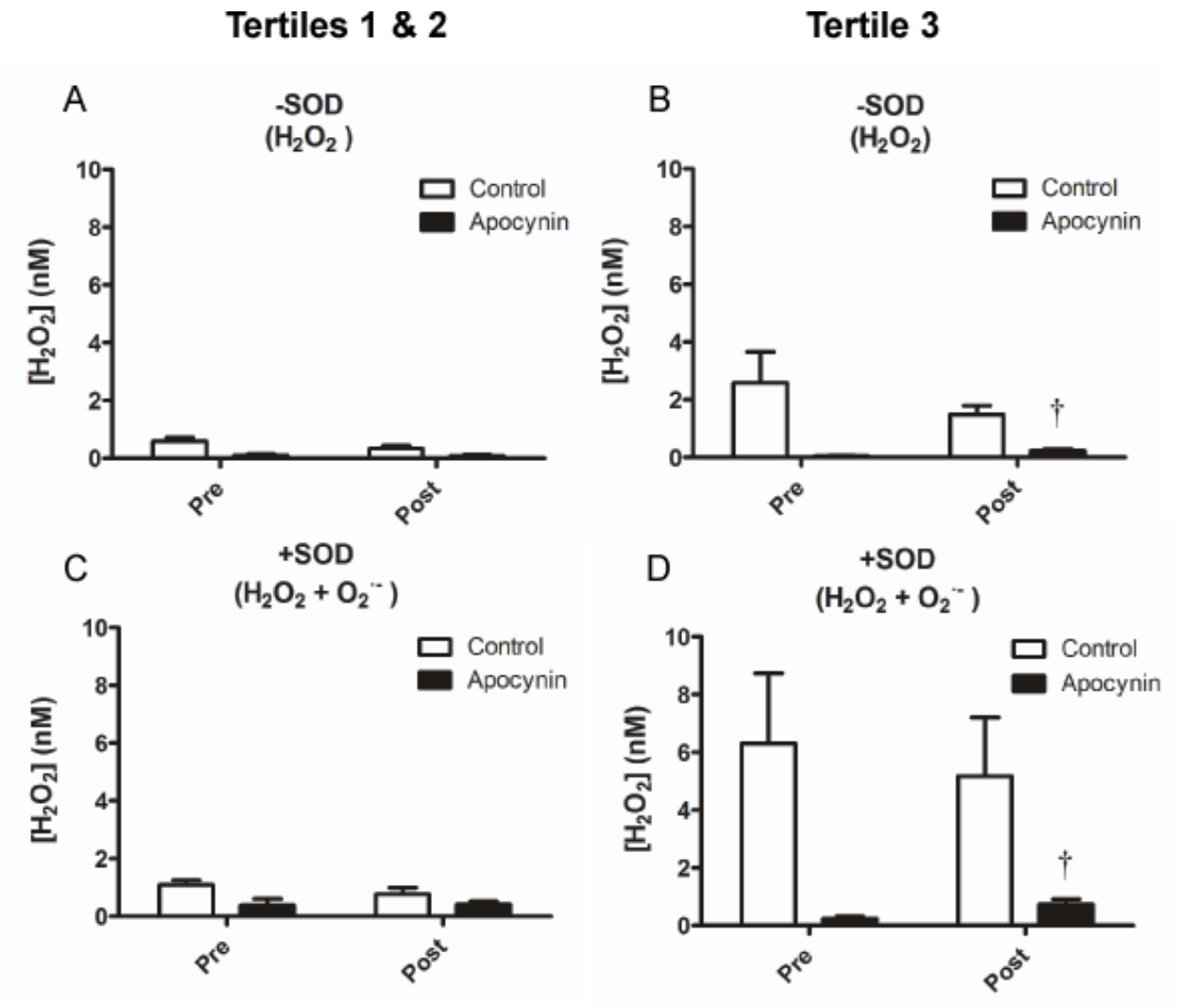

Figure 15 In vivo reactive oxygen species (ROS) production was measured before (Pre) and after (Post) the exercise training intervention, in the absence (control, open bars) and presence of apocynin (closed bars), an NADPH oxidase inhibitor. In vivo ROS production was measured without superoxide dismutase (SOD) added to the perfusate, which is indicative of H_2O_2 produced endogenously that crosses over the microdialysis membrane (A, C). ROS detected with SOD added to the perfusate is indicative of H_2O_2 produced endogenously that crosses over the microdialysis membrane in addition to H_2O_2 produced inside the microdialysis probe by SOD from superoxide that crosses over the microdialysis membrane (B, D). There were no significant

training induced changes in ROS production in either tertile 1 and 2 or tertile 3. There was a significant training induced increase in H_2O_2 (B) and combined H_2O_2 and superoxide (D) that was not blocked by apocynin, suggesting an increase in non-apocynin inhibitable ROS production with exercise training. † $P < 0.05$ vs. pre-training apocynin. Values are mean ± SEM for 5 subjects in tertiles 1 and 2, and 4 subjects in tertile 3.

Effects of Exercise Training on Microvascular Blood Flow Responses

There were no significant effects of the exercise training intervention on ACh-stimulated blood flow (P = 0.889; effect size = 0.079; Figure 16a) or ACh-stimulated blood flow in the presence of apocynin (P = 0.370; effect size = 0.460; Figure 16a) in tertiles 1 and 2. There was no significant training*apocynin interaction effect (P = 0.388) in tertiles 1 and 2. There was a significant increase in ACh-stimulated blood flow in the absence of apocynin (P = 0.034; effect size 1.19; Figure 16b) following the exercise intervention in tertile 3. There was no significant effect of exercise training on ACh-stimulated blood flow in the presence of apocynin (P = 0.932; effect size 0.091; Figure 16b). There was no significant training*apocynin interaction effect (P = 0.257) in tertile 3. There were no significant effects of exercise training on SNP-stimulated blood flow in tertiles 1 and 2 (P = 0.661; effect size = 0.197; Figure 17) or in tertile 3 (P = 0.302; effect size = 1.00; Figure 17).

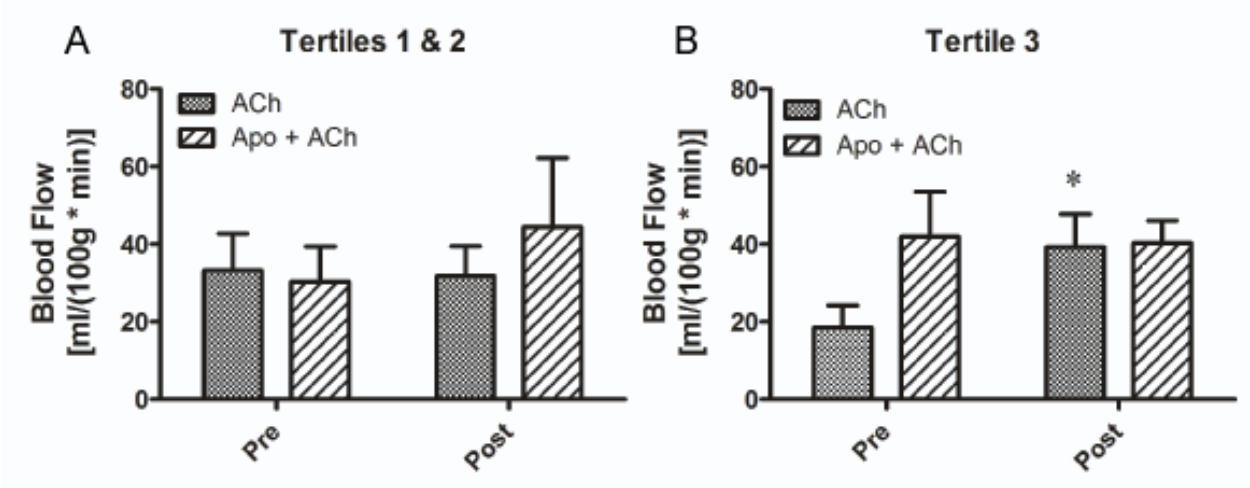

Figure 16 Microvascular endothelial function was assessed by acetylcholine (ACh)-stimulated blood flow before (Pre) and after (Post) an 8-week aerobic interval training intervention. ACh-stimulated blood flow assessments were made in the absence and presence of apocynin (Apo), an NADPH oxidase inhibitor. There were no significant effects of exercise training in tertiles 1 and 2 (A). There was a significant increase in ACh-stimulated blood flow in tertile 3 following exercise training (B). There were no significant effects of apocynin or training*apocynin interaction effects in either group. * $P < 0.05$ vs. pre-training. Values are mean ± SEM for 5 subjects in tertiles 1 & 2 and 4 subjects in tertile 3.

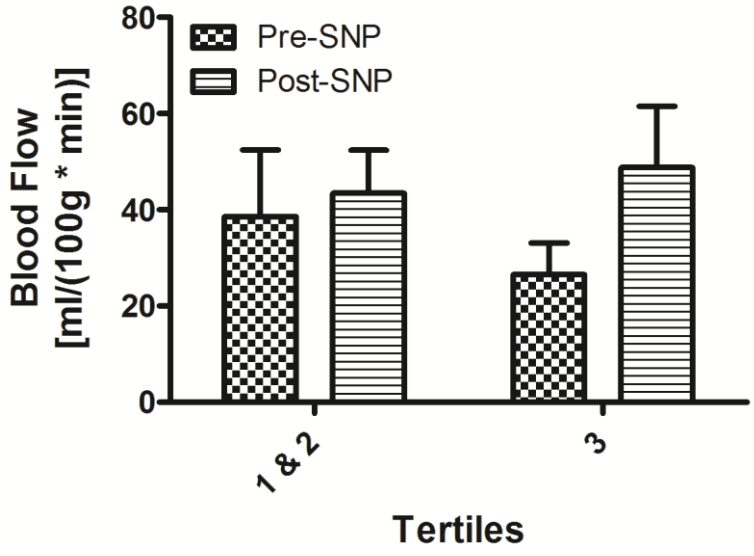

Figure 17 Endothelium independent dilation was assessed by sodium nitroprusside (SNP)-stimulated blood flow before (Pre) and after (Post) an 8-week aerobic interval training intervention. There were no significant effects of exercise training on SNP-stimulated blood flow in tertiles 1 & 2 or tertile 3. Values are mean ± SEM for 5 subjects in tertiles 1 & 2 and 4 subjects in tertile 3.

CHAPTER 5: DISCUSSION

In the collective work of this dissertation, we demonstrated that erectile dysfunction precedes coronary artery endothelial dysfunction in response to a high-fat, high-sucrose, Western pattern diet. Additionally, we demonstrated that diet-induced erectile dysfunction was partially reversed by acute apocynin or sepiapterin administration, while diet-induced coronary artery endothelial dysfunction was reversed by acute apocynin incubation. Furthermore, we demonstrated that an aerobic interval training intervention prevented both diet-induced erectile dysfunction and coronary artery endothelial dysfunction. We also developed a novel microdialysis method to detect *in vivo* extracellular reactive oxygen species production in human skeletal muscle, where we observed elevated reactive oxygen species production in individuals with a body mass index of 33 or greater. The increased reactive oxygen species production in these individuals was completely driven by NADPH oxidase. Furthermore, impaired skeletal muscle microvascular endothelial function was observed in the individuals with a body mass index greater than 33, which was also mediated by NADPH oxidase.

Addressing specific aim 1, we investigated the time course of development of erectile dysfunction and coronary artery endothelial dysfunction in response to a Western diet that is high in sucrose and fat derived from saturated and n-6 poly-unsaturated fatty acids. Rats demonstrated an impaired erectile response to electrical field stimulation of the cavernosal nerve near the major pelvic ganglion following eight weeks of the Western diet, where an impaired coronary artery relaxation response to ACh followed at 12 weeks. Rats on this Western diet did not demonstrate severe increases in body weight or adiposity. The 12-week WD fed rats developed significantly higher fasting blood glucose levels; however, there were no severe elevations of

insulin resistance or evidence of dyslipidemia. The 8-week and 12-week WD fed rats developed a trend for higher serum concentrations of HNE-adducts, suggesting elevated systemic oxidative stress. These findings indicate that chronic poor nutrition may supersede many of the traditional metabolic risk factors at predicting risk of ED and CAD development in this rodent model.

Consistent with the hypothesis that erectile dysfunction is an early indicator of future CAD, it is likely that a reduction in erectile function occurs prior to the onset of coronary endothelial dysfunction in the Zucker rat model, where obese Zucker rats (OZR) become diabetic and severely hyperlipidemic (132, 148, 149). In 17-18 week old lean (LZR) and OZR, there is a 4-fold increase in the media:lumen ratio in the penile arteries of OZR compared to LZR, where no difference was observed in the coronary arteries (149). This finding indicates that atherosclerotic development occurs in the penile artery prior to the coronary artery, and reflects the state of endothelial function as the vasodilatory response to ACh in the OZR is attenuated in the penile artery but not the coronary artery (149). Oltman et al. (148) found similar results as there was no difference in the coronary vasodilatory response to ACh between LZR and OZR at 16-24 weeks of age, where an impaired coronary vasodilatory response to ACh was apparent in OZR at 28-36 weeks of age. It is also likely that the reduction in penile artery endothelial dysfunction corresponds to erectile dysfunction as Wingard et al. (132) observed a reduction in ICP/MAP in OZR at 16-20 weeks of age. These findings in Zucker rats may translate to other models of obesity, as ten weeks of a 61.6% fat, lard-based diet attenuates ACh-stimulated vasodilatory response in the penile artery of Wistar rats (150), but not the coronary artery (151). In the present study, there were no substantial diet-induced differences in the coronary relaxation response to SNP, a direct NO donor and activator of the cyclic GMP mediated relaxation pathway. These findings are in contrast to those where the coronary dilatory response to SNP is

significantly augmented in response to a high-fat diet (151), suggestive of a sensitization to NO in diet-induce obesity. Considering the SNP response, the impaired relaxation response to ACh in the 12-week WD-fed rats is likely a dysfunction of the endothelium via impaired NO production as the smooth muscle retains a normal relaxation response to application of an NO donor.

Various high-fat or hypercholesterolemic diets have previously been employed to investigate erectile function, cavernosum dynamics, and biochemical characteristics. Feeding Sprague-Dawley rats a 2% cholesterol, 10% lard diet for 5-6 months has previously shown to attenuate the voltage-dependent erectile response, in addition to induction of cavernosal smooth muscle hyperplasia and a reduction in cavernosal endothelial cell content and nNOS positive nerve fibers (168-170). In a design similar to the present study, Xie et al (171) fed C57Bl6 mice a 45% fat diet for progressive durations and observed a progressive decline in ACh-mediated relaxation of cavernosal strips and cavernosal endothelial cell content with increased diet duration. Similarly, $ApoE^{-/-}$ mice fed a hypercholesterolemic diet for progressive durations displayed a progressive decline in cavernosal nNOS content, eNOS Ser^{1177} phosphorylation, capillary density, and ACh-mediated relaxation of cavernosal strips with increased diet duration (172). In addition to decreased cavernosal strip endothelium dependent dilation, feeding $ApoE^{-/-}$ mice a hypercholesterolemic diet has promoted increased lipid peroxidation and superoxide production, as well as decreased NO production from cavernosal tissue (173).

Erectile dysfunction and CAD both have the common underpinnings of endothelial dysfunction. This study supports the notion that erectile dysfunction may be an early marker of cardiovascular risk, as has been proposed in several review articles (174-177). In a large clinical

study, Thompson et al (178) found that the association between ED and CVD was nearly identical to the risk associated with current smoking or family history of myocardial infarction, both of which have long been established as CVD risk factors. Presentation of ED may provide an opportune time-point for clinician intervention. Long-term weight loss interventions through increased physical activity and/or caloric restriction have proven successful at improving erectile function in obese men (123, 179). While it is logical that such lifestyle interventions initiated at the onset of ED would postpone, or perhaps prevent CAD development, future studies are necessary to establish these relationships.

Addressing specific aim 2, we investigated the impact of NADPH oxidase and indicators of BH_4 content associated eNOS uncoupling on erectile dysfunction and coronary artery endothelial dysfunction in response to a 12 week Western diet that is high in sucrose and fat derived from saturated and n-6 poly-unsaturated fatty acids. Furthermore, we investigated the efficacy of an aerobic interval training intervention on prevention of diet-induced vascular dysfunctions. Rats demonstrated an impaired erectile response to electrical field stimulation of the cavernosal nerve near the major pelvic ganglion, and an impaired coronary artery relaxation response to ACh following the chronic Western diet, both of which were prevented by the exercise intervention. Inhibition of NADPH oxidase with apocynin significantly improved erectile function and coronary artery endothelial function in the Western diet-sedentary group, while having no impact in the control diet or exercised groups. In contrast, stimulation of intracellular BH_4 production with sepiapterin significantly improved erectile function in the Western diet-sedentary group, but had no effect on coronary artery endothelial function. Sepiapterin treatment attenuated coronary artery endothelial function in the control diet-sedentary group, while having no effect on the Western diet or exercised groups. These findings

indicate that erectile function and coronary artery endothelial function are commonly impaired by NADPH oxidase in a 12 week Western diet-induced obesity model. However, the sepiapterin data indicate that eNOS uncoupling has a greater effect on erectile dysfunction than coronary artery endothelial dysfunction in the Western diet-induced obesity model.

Several studies have demonstrated impaired aortic endothelial function in high-fat diet-induced obese rats (39, 153), and mice (155), as well as coronary artery endothelial dysfunction of mice (180) and pigs (6, 156). Elevated oxidative stress has been documented in these vascular tissues by increased levels of nitrotyrosine (39), while treatment with superoxide dismutase (SOD) (155) or the superoxide scavenger TEMPOL (180) were shown to reverse high-fat diet-induced endothelial dysfunction. Furthermore, the NADPH oxidase subunits $p47^{phox}$ and $gp91^{phox}$ were found to be elevated in these vascular tissues (153, 155, 180).

Similar to other vascular beds, oxidative stress has been demonstrated in cavernosal tissue of $LDLR^{-/-}$ mice fed a hypercholesterolemic diet (138), cholesterol fed rabbits (145), and high-fat diet fed pigs (144). Increased cavernosal markers of oxidative stress have also been observed in other models that demonstrate ED, such as streptozotocin (STZ)-induced diabetic rats (129, 141, 142, 181), and angiotensin II supplemented hypertensive rats (134). Oxidative stress is a likely contributor to ED, as treatment with various antioxidants (141, 142) or transfection of SOD (129) normalize markers of cavernosal oxidative stress and augments cavernosal vasorelaxation in STZ-diabetic rats. Similarly, elevated NADPH oxidase has been implicated in the pathogenesis of ED. Elevated expression of NADPH oxidase subunits have been found to be elevated in cavernosal tissue of angiotensin II supplemented hypertensive rats (134), STZ-induced diabetic rats (181), and $LDLR^{-/-}$ mice fed a hypercholesterolemic diet (138).

Furthermore, addition of apocynin to drinking water blunts NADPH oxidase subunit expression and restores the voltage dependent erectile response in hypertensive rats (134) and hypercholesterolemic mice (138), while apocynin incubation of isolated cavernosal strips of hypercholesterolemic rabbits partially restores the relaxation response to an ACh analog (145). The present study demonstrates the impact of NADPH oxidase mediated oxidative stress on obesity associated ED, as acute NADPH oxidase inhibition partially reverses ED.

One potentially vasodestructive consequence of elevated NADPH oxidase activity is the quenching of NO by superoxide, resulting in the production of peroxynitrite. Further, peroxynitrite may then deplete intracellular BH_4 levels, by direct oxidation of BH_4 to BH_2 (92). Adequate presence of BH_4 is a critical requirement for normal eNOS functioning, which acts to stabilize eNOS dimers and allows for the oxidation of L-arginine and the subsequent generation of NO (36). Destabilization of eNOS dimers through BH_4 depletion results in "eNOS uncoupling", where electron flow from NADPH through eNOS to O_2 is not inhibited, but results in the formation of superoxide rather than NO (36). The resulting situation is a potential vicious cycle where NADPH oxidase activity is elevated in metabolic syndrome conditions, which promotes endothelial dysfunction through NO quenching and eNOS uncoupling, and further endothelial dysfunction via progressive loss of NO bioavailability and superoxide production from eNOS. Feeding Yucatan miniature swine a 46% fat diet for 24 weeks has shown to increase penile thiobarbituric acid reactive substances (TBARS) and decrease the penile eNOS dimer:monomer ratio, demonstrating an association between an enhanced oxidative burden in the penis and eNOS uncoupling (144). NADPH oxidase induced eNOS uncoupling has been demonstrated in hypertension, where aortas of mice made hypertensive by deoxycorticosterone acetate-salt (DOCA-salt) exhibit depleted BH_4 levels and increased eNOS derived superoxide

production, while $p47^{phox-/-}$ mice maintain BH_4 levels and resist the upregulation of eNOS derived superoxide production associated with DOCA-salt hypertension (100). Furthermore, $LDLR^{-/-}$ mice fed a hypercholesterolemic diet demonstrate a decreased cavernosal dimer:monomer ratio, while drinking water supplementation of apocynin restores the voltage dependent erectile response and the cavernosal eNOS dimer:monomer ratio (138). While we cannot be certain that NADPH oxidase induced eNOS uncoupling was the predominant mechanism promoting erectile dysfunction in response to the 12 week Western diet, NADPH oxidase inhibition with apocynin and BH_4 stimulation with sepiapterin both augmented the voltage dependent erectile response to a similar degree. It should be noted that sepiapterin stimulates intracellular BH_4 production, which may act as an antioxidant (36). Thus, in the absence of NOS dimer:monomer measurements, we cannot be certain that the reversal of ED with sepiapterin treatment is driven by enhanced NOS coupling rather than oxidant scavenging.

Contrary to the voltage dependent erectile response, sepiapterin treatment did not improve coronary artery endothelial dysfunction in the Western diet-sedentary rats. 10 μM sepiapterin treatment has previously restored endothelial function in aortic segments of $ApoE^{-/-}$ mice (92). Furthermore, oral administration of sepiapterin has been found to augment left ventricular function in STZ-induced diabetic mice (182), while intravenous administration of sepiapterin restores the cardioprotective effect of ischemic preconditioning that is completely blocked by severely hyperglycemic conditions (183). However, investigations on the effect of sepiapterin treatment specifically on coronary artery endothelial function has been limited. 100 μM sepiapterin has previously been found to attenuate endothelial dependent vasorelaxation responses in canine middle cerebral arteries (184) and rabbit aortas (38), which agrees with the findings of the present study in which 10 μM sepiapterin attenuated ACh-stimulated

vasorelaxation in coronary artery segments of control diet-sedentary rats. It has previously been suggested that sepiapterin supplementation to normally functional eNOS could stimulate eNOS-dependent superoxide generation, resulting in potential for attenuation of endothelial function (185). Additionally, the hypothesis of NADPH oxidase induced eNOS uncoupling suggests that NADPH oxidase activity is increased prior to any depletion of BH_4 and resultant eNOS uncoupling (186). The Western diet used in the present study has previously been demonstrated to induce erectile dysfunction more rapidly than coronary artery endothelial dysfunction (187). Thus, it is probable that NADPH oxidase activity is also increased in the cavernosum prior to the LAD coronary artery. The findings addressed in specific aim 1 suggest that 12 weeks of exposure to the Western diet represents a very early time point in development of CAD in this rat model. These Western diet-sedentary rats demonstrated mild elevations in adiposity and blood glucose levels, with no substantial alterations in blood lipids or insulin sensitivity. Thus, it is possible that prolonging the dietary intervention may produce a situation similar to that in the penis, where functionality is augmented by either apocynin or sepiapterin treatment, as prior studies demonstrating increased vasoprotection or cardioprotection with sepiapterin treatment have utilized overtly hypercholesterolemic, hyperglycemic, or diabetic models (92, 182, 183). This study is in agreement with prior investigations, where apocynin has been shown to reverse endothelial dysfunction in coronary arteries of insulin resistant obese Zucker rats (188), type II diabetic mice (189), and high-fat diet induced obese mice (180). This study demonstrates that NADPH oxidase is an important mechanism of early coronary artery endothelial dysfunction, where endothelial function does not appear to be impaired due to attenuated BH_4 availability.

The potential cardioprotective benefits of aerobic exercise are well documented (46). However, investigations on the impact of exercise interventions on erectile function are rather

limited. Obese men with ED who received lifestyle advice regarding diet and physical activity have reported improved erectile function (123), while treadmill exercise plus caloric restriction has been shown to improve erectile function in aging and hypertensive rats (190). Additionally, physical activity has been shown to augment the efficacy of phosphodiesterase 5 inhibition on ED reversal (191). There are previous implications for a protective effect of exercise through eNOS coupling, as treadmill exercise of pigs has shown to preserve the cavernosal eNOS dimer:monomer ratio that is attenuated in response to a hypercholesterolemic diet (144). Interestingly, a similar intervention has proven to preserve coronary artery endothelial function through a NO-mediated mechanism in this model (156). Importantly, exercise has shown to improve endothelial function in internal mammary artery segments and attenuate NADPH oxidase subunit expression in CAD patients (112). Similar to the present study, the apocynin-induced augmentation of coronary artery endothelial function in high-fat diet fed mice is lost with the addition of voluntary wheel running (180). Aerobic interval training may be a particularly advantageous mode of intervention in the metabolic syndrome. Interval training protocols similar to that utilized in the present study have been shown to substantially augment flow-mediated dilation and aerobic capacity in human metabolic syndrome patients (47), heart failure patients (48), and CAD patients (192), as well as rodents bred to have a low aerobic capacity (49). In the present study, we demonstrate that aerobic interval training prevents Western diet-associated erectile dysfunction and coronary artery endothelial dysfunction.

Addressing specific aim 3, we investigated the impact of increasing body mass index on extracellular reactive oxygen species production and microvascular endothelial function. Subjects in the highest tertile of BMI demonstrated significantly elevated levels of combined H_2O_2 and superoxide production (ROS production). Remarkably, ROS detected in the presence

of apocynin was not different in the highest BMI tertile compared to the lowest BMI tertile. This finding suggests that the elevation in detectable extracellular ROS associated with high BMI was completely due to elevated NADPH oxidase activity that was inhibitable by apocynin. In addition to elevated ROS production, subjects in the highest BMI tertile also demonstrated impaired microvascular endothelial function relative to both other tertiles. Similar to both erectile function and coronary artery endothelial function, impaired microvascular endothelial function in the peripheral vasculature was improved by NADPH oxidase inhibition with apocynin. Preliminary data suggests that an 8-week aerobic interval training intervention may reverse microvascular endothelial dysfunction in the highest BMI tertile. However, the exercise intervention does not appear to alter extracellular ROS production in the highest BMI tertile, suggesting that any improvements in microvascular endothelial function associated with exercise training are likely due to mechanisms other than ROS production.

A multitude of studies have utilized microdialysis to detect reactive oxygen species in rodent brain in various models of neurological disease or conditions of environmental stress (193-197). The vast majority of these studies have utilized perfusion of salicylate, which reacts with the hydroxyl radical to form 2,3-dihydroxybenozoate which is detectable with high performance liquid chromatography or gas-chromatography/mass-spectroscopy (194-197). Fewer investigations have utilized microdialysis perfusion of an electron spin resonance trap to detect non-specific ROS (193, 195). Notably, $gp91^{phox}$ knockout has been shown to prevent lipopolysaccharide-induced ROS production in the brain (193), suggesting that NADPH oxidase is a prominent source of septic shock associated ROS production. Fewer studies have utilized microdialysis to detect ROS outside of the brain. Dihydroethidium and salmon DNA have been perfused to detect superoxide production in the renal medulla of rats (198, 199). Extracellular

superoxide production in the renal medulla elevated in the salt-sensitive hypertension model used, and was alleviated by seven days of apocynin perfusion of the renal medulla (199). Other prior investigations have utilized microdialysis to measure ROS in the extracellular fluid of skeletal muscle (200-205). These investigations have focused on measuring $O_2^{\bullet-}$ release from contracting skeletal muscle, utilizing perfusion of cytochrome c and analysis of reduced cytochrome c as an indicator of $O_2^{\bullet-}$ production. The majority of these investigations have utilized microdialysis in the gastrocnemius of anesthetized rodents (200, 202-205), while Hellsten et al. performed microdialysis in the vastus lateralis of physically active, healthy humans (201). Cytochrome c can be reduced by the hydroxyl radical or nitric oxide (50) and can be oxidized by peroxynitrite (206). The amplex ultrared method utilized in the present study offers several advantages in that measurements can be made simply with a portable fluorometer as opposed to chromatography based methods, there is no need to perfuse DNA, and there is no potential for increased fluorometric signal induced by nitric oxide. This study is the first to document increased *in vivo* extracellular ROS production in a human pathological state.

NADPH oxidase activity, $p22^{phox}$, $p47^{phox}$, and $p67^{phox}$ expression have been shown to be elevated in internal mammary arteries of human type II diabetic patients (31), and coronary artery segments of human CAD patients (32), compared to non-diabetic patients, and patients without CAD, respectively. Additionally, $p67^{phox}$ mRNA expression is elevated in saphenous veins of heart failure patients undergoing a coronary artery bypass graft. Similar to the present study, treatment with apocynin has been shown to improve nitric oxide mediated vasodilation and attenuated ROS production in small arteries removed from visceral adipose tissue of obese patients undergoing bariatric surgery (207). Silver et al have demonstrated a positive association between human vein endothelial cell $p47^{phox}$ expression and both BMI and adiposity (208).

Induction of NADPH oxidase activity with increasing adiposity is likely complex and multifactorial. Treatment of cultured smooth muscle cells with either angiotensin II (209) or leptin (210) stimulates NADPH oxidase activity. Likewise, incubation with the inflammatory adipokine visfatin stimulates NADPH oxidase activity in endothelial cells and impairs coronary artery endothelium dependent dilation (211). Three days of intraperitoneal injection of C-reactive protein (CRP) induces an increase in aortic $p47^{phox}$ membrane translocation, while injection or isolated vessel incubation with CRP impair mesenteric artery ACh-stimulated vasorelaxation (212). Similarly, three days of intraperitoneal injection of TNF-α induces an increase in $p22^{phox}$ and $p40^{phox}$ mRNA expression (188). Thus, NADPH oxidase induced endothelial dysfunction associated with increased BMI and adiposity is likely mediated in part through the inflammatory cascade, while previous findings of increased NADPH oxidase activity or subunit expression in obesity associated pathological states are congruent with the findings of the present study.

Previous studies suggest that aerobic exercise reverses endothelial dysfunction through an attenuation of NADPH oxidase (107-109, 112, 113). The findings of the present study suggest that NADPH oxidase inhibition reverses microvascular endothelial dysfunction in the highest BMI tertile prior to exercise training, while having no effect following exercise training. Due to the low statistical power, the exercise training results of the present study must be interpreted with extreme caution. There was a large, positive effect-size of exercise on ACh-stimulated blood flow in the highest BMI tertile. However, there was also large, positive effect-size of exercise on SNP-stimulated blood flow in the highest BMI tertile. Thus, any apparent reversal of microvascular endothelial dysfunction by exercise training may be due to improved signaling of the cGMP pathway rather than improved capacity for NO production. There was a small effect-size of exercise training on decreased ROS production in the highest BMI tertile, suggesting that

the 8-week exercise intervention does not alter ROS production, and likely does not alter NADPH oxidase activity itself. There was a large effect-size for a post-exercise training increase in non-apocynin inhibitable ROS production. This finding suggests that sources of extracellular ROS other than NADPH oxidase are elevated following the exercise intervention. Various exercise conditions have increased xanthine oxidase activity in rodent muscle (205, 213-215), indicating that the contribution of xanthine oxidase to extracellular ROS may increase with exercise training. Aside from redox regulation, exercise training may increase eNOS activity via increased HSP-90 association (111) or increased Ser^{1177} phosphorylation (106, 216, 217), which may be independent of oxidative stress.

In summary, these studies demonstrate that NADPH oxidase is a common mechanistic link between erectile dysfunction and coronary artery endothelial dysfunction in rats fed a high-fat high-sucrose diet, and peripheral endothelial dysfunction in a high BMI human population. In the face of a worldwide obesity epidemic, novel approaches to treatment and prevention of CAD are increasingly necessary. These studies demonstrate that erectile dysfunction precedes the development of CAD in response to a Western diet, which supports the notion that ED may be an early marker of cardiovascular risk (174-177). Several studies have further suggested that peripheral endothelial function can be used for cardiovascular risk assessment (218-221). Access to human penile tissue for mechanistic studies of erectile function is extremely limited. However, peripheral skeletal muscle beds provide a dense microvascular network where invasive studies conducted under local anesthesia provide a vascular bed in which mechanistic studies can be conducted in humans that may be predictive of coronary vascular function. These studies describe a novel method by which extracellular ROS production can be measured alongside assessment of microvascular endothelial function, and further allowing for localized

pharmacological manipulation. Interestingly, the Western diet-sedentary rats and the highest BMI tertile of humans demonstrated impaired endothelial function in the absence of substantial elevations in insulin resistance or any elevations in blood lipid profiles. These blood results indicate that investigation of peripheral endothelial function may supersede the traditional blood markers used for cardiovascular risk assessment. These combined findings further underscore the feasibility of potential therapeutic approaches aimed at treatment of ED and peripheral endothelial dysfunction and prevention of CAD development. These studies further demonstrate prevention of erectile dysfunction and CAD development with aerobic interval training in rats, while preliminary data suggest that aerobic interval training may reverse peripheral endothelial dysfunction in humans, providing a potentially practical means of decreasing risk of cardiovascular disease.

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APPENDIX A: IACUC – #Q303 – LETTER OF APPROVAL

East Carolina University

Animal Care and Use Commitee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

May 25, 2011

Christopher Wingard, Ph.D.
Department of Physiology
Brody 6N-98
ECU Brody School of Medicine

Dear Dr. Wingard:

Your Animal Use Protocol entitled, "Investigating Erectile Dysfunction as a Coronary Artery Disease Risk Factor through NO and NADPH Oxidase, and Prevention by Exercise Training" (AUP #Q303) was reviewed by this institution's Animal Care and Use Committee on 5/25/11. The following action was taken by the Committee:

"Approved as submitted"

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Scott E. Gordon, Ph.D.
Chairman, Animal Care and Use Committee

SEG/jd

enclosure

APPENDIX B: UMCIRB #11-0262 LETTER OF APPROVAL

EAST CAROLINA UNIVERSITY
University & Medical Center Institutional Review Board Office
1L-09 Brody Medical Sciences Building• 600 Moye Boulevard • Greenville, NC 27834
Office **252-744-2914** • Fax **252-744-2284** • **www.ecu.edu/irb**

TO: Justin LaFavor, MS, ECU, Department of Exercise and Sport Science, Mailstop 158

FROM: UMCIRB KwB

E MAILED 6-1-11 PI & Respfac

DATE: May 26, 2011

RE: Full Committee Approval of a Study

TITLE: "Role of NADPH Oxidase Activity on Muscle Microvascular Endothelial Function in Human Obesity"

UMCIRB #11-0262

The above referenced research study was initially reviewed by the convened University and Medical Center Institutional Review Board (UMCIRB) on **04/27/2011.** The research study underwent a review and approval of requested modifications on **05/17/2011** by expedited review. The UMCIRB deemed this **American College of Sports Medicine** (pending award) sponsored study **more than minimal risk** requiring a continuing review in **12 months**. Changes to this approved research may not be initiated without UMCIRB review except when necessary to eliminate an apparent immediate hazard to the participant. All unanticipated problems involving risks to participants and others must be promptly reported to the UMCIRB. The investigator must submit a continuing review/closure application to the UMCIRB prior to the date of study expiration. The investigator must adhere to all reporting requirements for this study.

The above referenced research study has been given approval for the period of **04/27/2011** to **04/26/2012**. The approval includes the following items:

- Internal Processing Form – revised (UMCIRB receipt date 05/16/11)
- Protocol (dated 4/18/11)
- Informed consent – revised (dated 05/16/11)
- Medical history questionnaire
- 3 day food record with directions
- Recruitment flyer

The following UMCIRB members were recused for reasons of potential for Conflict of Interest on this research study: None

NOTE: The following UMCIRB members with a potential Conflict of Interest did not attend this IRB meeting: None

The UMCIRB applies 45 CFR 46, Subparts A-D, to all research reviewed by the UMCIRB regardless of the funding source. 21 CFR 50 and 21 CFR 56 are applied to all research studies under the Food and Drug Administration regulation. The UMCIRB follows applicable International Conference on Harmonisation Good Clinical Practice guidelines.

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