IN VIVO AND *IN VITRO* INTERACTIONS OF OXIDATIVE STRESS AND LAMINAR SHEAR STRESS ON VASCULAR ENDOTHELIAL GROWTH FACTOR-MEDIATED ENDOTHELIAL NITRIC OXIDE SYNTHASE ACTIVITY

A Dissertation
Submitted to
The Temple University Graduate Board

In Partial Fulfillment
of the Requirements for the Degree
DOCTOR OF PHILOSOPHY

By
Kathleen Sturgeon
August 2011

Examining Committee Members:
Michael D. Brown, Ph.D., Kinesiology, College of Health Professions and Social Work
Joon-Young Park, Ph.D., Kinesiology, College of Health Professions and Social Work
Victor Rizzo, Ph.D., Anatomy & Cell Biology, School of Medicine
Zebulon Kendrick, Ph.D., Kinesiology, Dean of Graduate School
Freda Patterson, Ph.D., Public Health, College of Health Professions and Social Work

ABSTRACT

Purpose: Translational research characterizing endothelial dysfunction and the progression of cardiovascular disease (CVD) is necessary for understanding the complex nature of multi-factorial diseases. Perhaps more important though, is understanding the compensatory and adaptive processes associated with regression of diseases and chronic oxidative stress. Vascular endothelial growth factor (VEGF) is an important protein in endothelial health and nitric oxide (NO) production. The purpose of this research was to examine changes in VEGF-mediated endothelial nitric oxide synthase (eNOS) activity under conditions of oxidative stress both *in vivo* and *in vitro*. **Methods:** The oxidative stress relationship involving plasma VEGF, NO, and hydrogen peroxide (H_2O_2) was assessed in sedentary, pre-hypertensive African American participants both (n=48) before and following (n=22) 6 months of aerobic exercise training (AEXT). *In vitro*, H_2O_2 exposure along with atherogenic, 4 dyne/cm^2, and athero-protective, 20 dyne/cm^2, levels of laminar shear stress (LSS) were used to characterize VEGF-mediated eNOS activity to gain insights into physiological signaling. **Results:** At baseline, VEGF levels increased with increasing blood pressure (BP) level while NO levels decreased from normotensive to hypertensive participants. H_2O_2 levels also trended upward with increasing BP level, and *in vitro* H_2O_2 was observed to decrease VEGF-mediated eNOS activity in a dose dependent manner. Following AEXT, participants were divided into groups relative to their BP change following the intervention. Participants that decreased their BP level

demonstrated a decrease in VEGF and H_2O_2 level. In addition, following 24 hrs of LSS at 20 dyne/cm^2, VEGF-mediated eNOS activity and VEGFR2 protein expression was significantly lower compared to 24 hrs of LSS at 4 dyne/cm^2. **Discussion:** Increased circulating levels of VEGF *in vivo* may be a compensatory mechanism. Endothelial dysfunction and progressive CVD may trigger such compensation. The adaptive response to exercise for its BP-lowering effects is systemic and encompasses many changes. These beneficial adaptations have likely alleviated the compensatory mechanism of elevated VEGF levels seen at baseline. Indeed, following 24 hrs of an athero-protective LSS level, VEGF-mediated eNOS activity was significantly lower compared to 24 hr of LSS at an atherogenic level. The difference in VEGF-mediated eNOS activity may be due, in part, to the decrease in VEGFR2 protein expression we observed under an athero-protective LSS level.

ACKNOWLEDGMENTS

This wasn't just a dissertation; it was a growing up process.

Dr Brown, thank you for giving me the opportunity to be part of your study and for fostering my intellectual development. I can't tell you how much I appreciate your time and dedication. I could not have asked for a better mentor to build the foundation for my professional development. The subtle lessons have been numerous and I know I will take them with me. Dr Park, thank you for taking me under your wing and teaching me not just molecular techniques but how to be a better scientist. There are questions I ask now and plans for problem solving that I would not have been equipped with if not for your instruction. Deb, my friend, my colleague, my sounding board, I could not have done this without you. Your unrelenting enthusiasm coupled with your energy and dedication has truly been inspirational. To everyone in the HyMAP and CvG Labs, thank you for your help, and your conscientious hard work.

Mom and Dad, thank you for encouraging me to always believe in myself. You've taught me so many valuable lessons just by example alone and I am fortunate to have such wonderful role models. Thanks for the all the little things the last few years that have added up to so much. Your love and support have been and continue to be invaluable. Jackie, you've been through this. I always knew I had someone in my corner I could scream my frustration to and they would get it. Thanks for letting me vent and also having great advice. I truly appreciate it and I am grateful that we'll always have each

other's back. Maura thank you for your interest in this work, you never failed as my personal cheerleader, making me smile. Barry, for all the ways you make me happy, relaxed, and loved, I thank you so much. You have brought a balance to my life that kept me out of research-induced funks. You have been understanding and thoughtful throughout this process and I'm so excited to continue building our lives together.

.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

REVIEW OF LITERATURE

Hypertension

Hypertension (HT) is a global health issue with a predicted 1.56 billion people worldwide diagnosed with HT by 2025[1]. In the United States alone, spending on health care reached $2.4 trillion in 2008[2], and the estimated direct and indirect cost of cardiovascular disease (CVD) in the United States during 2008 was $448.5 billion[3]. However, less than 1% of our nation's health spending is invested in prevention. Instead, virtually all of our nation's annual expenses for healthcare go to attempting to restore health, or some measure of it, once it is already lost[4].

In 2008, more American adults had HT (systolic blood pressure (SBP) >140 mmHg and diastolic blood pressure (DBP) >90 mmHg) or pre-hypertension (SBP 120-139 mmHg and DBP 80-89 mmHg) than ever before[5]. There are many factors that regulate cardiac output and peripheral resistance which together determine the level of blood pressure (BP). Neural input comes from both the sympathetic and parasympathetic nervous systems and cardiac factors such as heart rate (HR) and contractility also play large roles. Also involved in BP regulation are endocrine factors such as renin, angiotensin, atrial natriuretic peptide (ANP), antidiuretic hormone (ADH), aldosterone, and mineralcorticoids. Therefore, the etiology of HT is very complex.

Another important factor in the regulation of BP is the endothelium. The cells that line the inside of blood vessels primarily maintain vascular homeostasis and therefore influence peripheral resistance. Endothelial dysfunction is the physiological disruption of normal endothelial processes. The term originated following the discovery by Furchgott and Zawadzki that acetylcholine requires the presence of endothelial cells (ECs) in order to relax vascular smooth muscle cells (VSMC)[6]. Initially called endothelium derived relaxing factor, the molecule released by the endothelium in response to acetylcholine was later identified as nitric oxide (NO)[7]. NO dilates blood vessels by stimulating soluble guanylyl cyclase and increasing cyclic guanosine monophosphate (cGMP) in VSMC[8]. NO also inhibits platelet aggregation[9] and adhesion[10], leukocyte adhesion[11], and VSMC proliferation[12].

Winquist et al. observed blunted relaxation of hypertensive rat aorta that was associated with decreased NO levels[13;14]. Similar findings in hyper-cholesterolemic rabbit arteries suggest that low NO levels could be a catalyst for atherosclerosis[15-17]. It has also been reported that hypertensives have impaired vasodilation in small arteries and arterioles. These findings have been demonstrated in coronary[18] and renal[19] circulation as well as the peripheral vasculature[20].

The current study focused on using a translational model of non-pharmacological treatment for the modulation of a component of the molecular etiology of HT. The endothelium is exposed to a dynamic environment. It is necessary to understand the complex interactions in this environment, and how these may manifest themselves at the phenotypic level *in vivo*.

Oxidative Stress

Among the many factors implicated in the pathophysiology of HT, compelling evidence indicates that reactive oxygen species (ROS) may be important[21]. Excess ROS are detrimental because of their ability to donate a free electron. Change in the redox state of a cell has the ability to affect multiple proteins and signaling pathways. In the endothelium, a shift in the redox balance can influence BP regulation mediated through endothelial dysfunction.

The major ROS molecules include: superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^\bullet$), and peroxynitrite ($ONOO^-$). ROS function as signaling molecules and are associated with regulating VSMC contraction, relaxation, and growth under physiological conditions[22;23]. When a person is healthy, ROS are produced at low concentrations. However, under pathological conditions, such as HT, ROS production is increased and can lead to endothelial dysfunction, increased contractility, VSMC growth and apoptosis, monocyte migration, lipid peroxidation, inflammation and decreased NO bioavailability[24-27].

Sources of ROS include but are not limited to: mitochondrial respiration, arachidonic acid pathway enzymes, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, and NO synthase[28]. In the vasculature, the predominant source of ROS is NADPH oxidase. This multi-subunit enzyme catalyzes the production of O_2^-**.** Superoxide is highly reactive and can donate its extra electron to NO. The addition of an electron to NO yields $ONOO^-$ and decreases the bioavailability of NO[29]. This is just one way in which NO bioavailability can be decreased.

Endothelial Nitric Oxide Synthase Uncoupling

Uncoupling of endothelial nitric oxide synthase (eNOS) occurs when the flow of electrons in the eNOS enzyme structure is disturbed. Normally, eNOS binds (6R-)5,6,7,8-tetrahydrobiopterin (BH_4) to its oxygenase domain along with molecular oxygen and the substrate L-arginine. In order for NO to be produced, NADPH, flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD) are also bound to the monomers. eNOS first hydroxylates L-arginine to N-hydroxy-L-arginine and then oxidizes that molecule to L-citrulline and NO[30]. However, when the eNOS dimer is disrupted the enzyme becomes a dysfunctional O_2^- generating enzyme that contributes to vascular oxidative stress. One way that the dimer can be disrupted is through ROS oxidizing the zinc thiolate structure in eNOS and thus causing O_2^- production by eNOS.

A lack of substrate or cofactors for eNOS will also decrease bioavailability of NO[31]. Disruption of normal NO formation can occur from a lack of BH_4. This essential cofactor of eNOS can become oxidized by ROS causing its levels to deplete[32]. Also, although normal plasma concentrations of eNOS substrate L-arginine are well above the amount necessary for eNOS function, L-arginine supplementation has shown beneficial effects[33]. This could be due to L-arginine's direct radical scavenging abilities[34].

Antioxidants

When ROS levels exceed the antioxidant capacity of the cellular environment, oxidative stress occurs. The most studied cellular enzymatic antioxidants are the enzymes superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx)[35;36]. SOD converts O_2^- into H_2O_2. However, H_2O_2 is still mildly reactive itself and can also be

broken down into OH^- via the Fenton reaction. Yet, SOD is considered an antioxidant as the production of H_2O_2 is an important step in removing O_2^-. The antioxidant primarily responsible for removing H_2O_2 is catalase. It is an enzyme that catalyzes the decomposition of H_2O_2 into H_2O and O_2[37]. Finally, GPx is responsible for reducing lipid hydroperoxides to their corresponding alcohols and also for the break down of H_2O_2 into water[38;39].

The antioxidant system is a critical mediator of the health of the endothelium. The level of ROS present in ECs or the vascular system can influence signaling pathways. Therefore the ratio of oxidant production to antioxidant capacity is fundamental to the level of oxidative stress and progression of chronic diseases. Systemic and sustained elevation of ROS in the vasculature is detrimental to endothelial function as changes in signaling pathways can be altered. The research done characterized the interaction of ROS and other critical biomarkers of endothelial health *in vivo*, and examined how elevated ROS levels and an atherogenic environment impacted a necessary pathway of signaling in ECs.

Vascular Endothelial Growth Factor

An important molecule in the homeostasis of the endothelium is vascular endothelial growth factor (VEGF). Though encoded from a single VEGF gene located at 6p12[40] , there are at least 7 VEGF-A homodimeric isoforms (VEGF-A 121, 145, 148, 165, 183, 189, and 206)[41-43]. Of the 8 coding exons, exons 1 to 5 are conserved in all the monomers. Exons 6 and 7 encode heparin-binding domains[44], but alternative splicing in these regions determines the amino acids present and thus the solubility of the isoform[45].

VEGF-A holds a central position in the angiogenic process[46;47]. Hypoxia and the need for greater oxygen delivery is the physiological stimulus for new capillary growth[48]. Therefore, VEGF's transcription is highly induced by hypoxia and hypoxia inducible factor-1α (HIF-1α) is a potent transcription factor of VEGF[49-51]. Yet, under normoxic conditions HIF-1α is degraded by the von Hippel-Lindau (VHL) ubiquitylation complex[52].

Once VEGF-A is secreted, it can bind to its tyrosine kinase receptors, vascular endothelial growth factor receptor 1 (VEGFR1) or vascular endothelial growth factor receptor 2 (VEGFR2). VEGFR1 was previously known as fms-related tyrosine kinase 1 (Flt-1) and VEGFR2 was previously know as fetal liver kinase-1 (Flk-1) in mice and kinase insert domain-containing receptor (KDR) in humans. VEGFR2 has been implicated as the primary receptor which mediates VEGF signaling in ECs[53]. VEGF-A can also recognize Neuropilin-1 (NP-1) and bind to this transmembrane protein[54]. NP-1 has no signaling function, though it is co-expressed with the VEGF receptors. It may serve to localize VEGF to the area of its receptors.

Vascular Endothelial Growth Factor Receptor 2

This tyrosine kinase receptor has seven immunoglobulin–like domains in the extracellular region and an intracellular region of 70 amino acids[55]. The protein is coded by the KDR gene located at 4q11q12. The initial 150kDa protein is then processed and glycosylated to a 230kDa protein on the cell surface[56].

Activation of VEGFR2 is ligand stimulated and also shear stress stimulated[57]. After autophosphorylation of the tyrosine kinases, a number of src homology 2 domain

proteins and adaptor proteins associate with the complex[58]. EC survival is promoted through the phosphoinositide-3 kinase (PI3K)/protein kinase B(PKB/Akt) pathway[59] and further anti-apoptotic mechanisms[60;61]. VEGF also stimulates EC proliferation and DNA synthesis via the extracellular signal-regulated kinases (ERKs)[62;63]. Migration of ECs is also essential in angiogenesis and VEGF initiates this movement by the phosphorylation of focal adhesion kinase (FAK)[64].

Vasoactive Properties

In relation to vascular tone, it has been observed that VEGF induces NO dependent relaxation[65]. This relaxation is due to an increase in NO synthesis[66] through an increase in cytosolic calcium, and through elevated expression of eNOS[67]. The initial increase in eNOS activity by VEGF is through calcium influx in the cytosol. Though not entirely understood, it is believed that transient receptor potential cation channels (TRPCs) may play a role[68] in VEGF stimulated calcium increases. Increased calcium concentration increases the rate of calcium binding to calmodulin[69]. The calcium/calmodulin complex then changes conformation and exposes hydrophobic regions which allow for direct binding to eNOS[70]. Once the complex has found its complementary region on eNOS, the conformational changes initiate electron transfer from flavins to the heme moieties. VEGF also activates eNOS via Akt phosphorylation and AMP-activated protein kinase (AMPK) phosphorylation[71;72]. These vasoactive pathways were demonstrated in a clinical trial by Eppler et al. in which rapid infusion of VEGF resulted in a rapid fall (8-12mmHg) in mean arterial pressure (MAP)[73].

A delayed effect of VEGF-induced increases in NO is seen through increased transcriptional activity of eNOS via the PI3K/Akt pathway[74]. VEGF activation of its receptor, VEGFR2, also leads to the phosphorylation of cellular sarcoma tyrosine kinase (c-Src) and downstream production of the vasodilator prostacyclin (PGI-2)[75]. Also demonstrating the importance of VEGF in the vasculature are the recent observations of clinical cancer trials. Inhibition of VEGF signaling by anti-VEGF or anti-VEGFR cancer therapies has a significant side effect of advanced HT (stage-2 and stage-3 HT)[76-79]. Blocking VEGF signaling has a significant vascular effect. Abolition of VEGF signaling during anti-VEGF therapies may have beneficial effects of abrogating the EC proliferation and migration pathways of VEGF. However, it also may reduce NO bioavailability and EC survival.

While inhibition of VEGF signaling in cancer therapy causes HT, essential hypertensives demonstrate significantly elevated circulating plasma VEGF in comparison to age-matched normotensives[80]. This elevation was also seen in untreated stage-2 hypertensives[81] and in hypertensives of the Anglo-Scandinavian Cardiac Outcome Trial[82]. Plasma VEGF is also reported to be significantly elevated in individuals with hyperlipidemia[83], congestive heart failure[84], obesity[85], and peripheral artery disease[86]. In addition, VEGF also plays a large role in controlling vascular permeability. In a recent study by Viazzi et al., vascular permeability was associated with occurrence of sub-clinical cardio-renal primary HT[87]. It has been demonstrated that exquisite control of glomerular VEGF is necessary[88] and dysregulation caused by HT may compound the condition.

The elevation of VEGF seen in the above pathologies may be a compensatory mechanism. Given the vasoactive properties of VEGF, an increase in NO production would be favorable to alleviate decreased NO bioavailability under stress. Using a beneficial stimulus such as exercise to decrease oxidative stress *in vivo*, the study investigated the possible alleviation of this compensatory mechanism. The compensatory mechanism was also examined *in vitro* by creating conditions that elicit an atherogenic or athero-protective protein expression profile.

Exercise

Physical activity and exercise are overwhelming factors that induce normal physiological function within the vasculature[89]. Aerobic exercise training (AEXT) is a recommended lifestyle modification for hypertensive individuals and AEXT is necessary for the sustained health of an individual. Exercise can lower SBP by 6 to10 mmHg, DBP by 4 to 8 mmHg, and restore endothelial function via increased levels of laminar shear stress (LSS)[90-93]. It has been demonstrated that AEXT decreases levels of oxidative stress biomarkers[94;95], increases NO bioavailability [96-102], and increases antioxidant enzyme (SOD, GPx, catalase) gene expression [103-105].

Signal transduction of physiological LSS in ECs is necessary for physiological function[106]. Three decades of research has validated that pathological (low and or oscillatory) shear stress is a powerful local stimulus for atherogenesis. The vessel wall at bifurcations is geometrically inclined to encounter a lower magnitude of shear stress, and also multi-vectors of shear stress[107]. However, unidirectional shear stress at elevated physiological levels as discussed below is very beneficial.

Laminar Shear Stress

Exercise, via increased LSS, decreases oxidative stress and endothelial dysfunction. Mechanosensors on the endothelium transduce the exercise-induced increase in LSS to the ECs. The sensors activate biochemical pathways such as rat sarcoma (Ras)/rapidly accelerated fibrosarcoma (Raf)/mitogen activated protein kinase/extracellular signal regulated kinase (MEK), and c-Src[108;109]. These pathways in turn activate transcription factors such as: Nuclear Factor-$_k$B (NF_kB), c-Jun N-terminal Kinase (JNK), ERK, Krüppel-like Family (KLF), Activator Protein-1 (AP-1), and Nuclear erythroid-2 related factor 2 (Nrf2), which modify gene expression[110-112].

One of the key genes in HT modified by increased LSS is eNOS. Sessa et al. observed an enhanced expression of eNOS in the vasculature of exercise trained dogs[113]. This correlated with increased NO released from LSS stimulated vessels and cultured ECs[114]. This kinesiogenomics regulation produces a healthy endothelium[115]. *In vitro* use of elevated LSS such as 30 dyne/cm^2 over 24 hr has been shown to reproduce cellular changes characteristic of healthy *in vivo* endothelium[116].

Shear stress ranges from 1 to 6 dyne/cm^2 in the venous system and from 10-70 dyne/cm^2 in the arterial system[117]. LSS at a magnitude greater than 15 dyne/cm^2 induces endothelial quiescence and an athero-protective gene expression profile; in other words, a healthy endothelium. Low LSS ($\leq$4 dyne/cm^2), which is prevalent at atherosclerosis-prone sites, stimulates an atherogenic phenotype.

The beneficial magnitudes of LSS are further exemplified in a study by Duerrschmidt et al. in which they applied LSS to human umbilical vein endothelial cells

(HUVECs) at 1, 5, 10, 15, 30, and 50 dyne/cm^2 for 24 hr [118]. They then assessed NADPH oxidase expression in response to these different magnitudes of LSS. At LSS magnitudes of 1 and 5 dyne/cm^2, NADPH oxidase expression was actually greater than the control HUVECs that did not undergo LSS. This means greater O_2^- production and higher EC oxidative stress. At a LSS magnitude of 10 dyne/cm^2, NADPH oxidase expression was attenuated and equivalent to that of the control cells. It was not until LSS magnitudes reached 15, 30, and 50 dyne/cm^2 that NADPH oxidase expression levels significantly reduced to a level that was half that of the controls.

The investigation used 20 dyne/cm^2 as an athero-protective magnitude of LSS. Using LSS as an alternative to a pharmacological stimulus more closely mimics *in vivo* interactions. An atherogenic condition was mimicked using LSS at 4 dyne/cm^2. VEGF stimulation and response of eNOS activity following this pre-conditioning characterizes the signaling pathway under atherogenic or athero-protective conditions.

Cellular Adaptations

Changes in endothelial protein expression due to exercise and the transduction of LSS result in beneficial adaptations. In HUVECs, up regulation of antioxidant, anti-inflammatory, anti-proliferative, and anti-apoptotic genes were observed after 24 hr of 10 dyne/cm^2 of LSS. In this genome wide association study, Wasserman et al. observed almost a 2-fold decrease in genes at 24 hr of LSS exposure relative to genes that were up-regulated at 6 hr of LSS[119]. While many genes are transiently regulated by LSS, it has been established independently of genome wide scans that expression of antioxidant and anti-inflammatory genes are significantly increased.

SOD and cyclooxygenase-2 (COX-2) mRNA are increased after 6hr of 10 dyne/cm^2 LSS[120]. SOD protein is also increased under longer LSS exposure[121]. Heme-oxygenase-1 (HO-1), glutathione S-transferase (GST) and NADPH:quinine oxidoreducatse 1 (NQO1) are antioxidant response element (ARE) genes and are up regulated by LSS as well[122]. Also, peroxiredoxins levels (which eliminate peroxides and $ONOO^-$) are increased under LSS conditions[123]. Adaption of the cellular environment to exercise via transduction of LSS is a hallmark of the beneficial nature of exercise. This adaption may be a mechanism by which oxidative challenges are mitigated and proper EC signaling can function.

VEGF Expression

Laminar shear stress influences VEGF expression. In a perfusion culture system Conklin et al. observed increased VEGF expression from porcine carotid arteries in response to 24 hr of low LSS (1.5 dyne/cm^2). This increase was relative to VEGF expression at physiological levels of LSS (15 dyne/cm^2)[124]. *In vivo*, such atherogenic conditions also produced increased levels of circulating VEGF. Again, the increase in VEGF levels may be a compensatory mechanism to activate the vasoactive pathways associated with VEGF signaling.

VEGFR2 Activation and Expression

LSS has been observed to activate VEGFR2 independently of VEGF[125]. Using 12 dyne/cm^2 for 60 min Chen et al. found autophosphorylation of VEGFR2 peaked at a 100% increase over static levels within 5 minutes. This activation however, decreased to basal levels by 60 min of LSS. Downstream activation of signaling molecules via LSS

activation of VEGFR2 showed an increase in phosphorylated Akt at serine 473 (pAkt(473) and phosphorylated eNOS at serine 1177 (peNOS(1177)[126]. Inhibition of the VEGFR2 kinase and antisense oligonucleotides directed at VEGFR2 both significantly attenuated LSS (30 min at 12 dyne/cm^2)-induced activation of Akt and eNOS. The same study also reported that inhibition of VEGFR2 significantly reduced flow-mediated NO-dependent arteriolar dilation *in vivo*[127].

Not only does LSS independently activate VEGFR2 but, Urbich et al. used gel mobility shift analysis to detect a Specificity protein -1 (Sp1) transcription factor binding site that is responsible for LSS-induced increase in VEGFR2 transcriptional regulation[128]. This group studied HUVECs under 15 dyne/cm^2 for 6, 16, and 24 hr to demonstrate a dose dependent increase in VEGFR2 expression.

H_2O_2

The *in vivo* generation of H_2O_2 is the end product of the enzymatic reaction between SOD and O_2^-. H_2O_2 has been used in many investigations as an oxidant challenge due in part to data that indicates H_2O_2 is more atherogenic than O_2^- [129-131]. H_2O_2 is longer lasting than O_2^- and is membrane permeable.

H_2O_2 originated by NADPH oxidase has the ability to propagate its own production in a vicious cycle that contributes to pathological signaling. *In vivo*, healthy elderly men were observed to have plasma H_2O_2 levels of 1.67 uM[132]. Lacy et al. documented H_2O_2 plasma production to range from .5 uM to 7 uM. This study also observed a significant increase in H_2O_2 levels with increasing SBP.[133]. Additionally,

H_2O_2 levels from individuals with past myocardial infarction were 7.15 uM[134], while patients with end stage renal disease exhibited plasma concentrations of 13 uM H_2O_2[135].

In vitro, Gonzalez-Pacheco et al. found that H_2O_2 concentrations over 8 uM for 30 min began to have cytotoxic effects on ECs and the cells demonstrated significant cell death at 60 uM of H_2O_2 (Figure 1)[136]. There have also been reports of HUVECs undergoing apoptotic cell death in H_2O_2 concentrations of 50 to 100 uM [137]. However, it appears this *in vitro* response can be attenuated by a high serum environment[138]. Several studies have used 50 uM bolus doses of H_2O_2 as a ROS stimulus[139;140]. Activated neutrophils can generate 10-1000 uM of H_2O_2 in local concentrations[141] while steady-state intracellular concentrations of H_2O_2 have been estimated in the nM range[142].

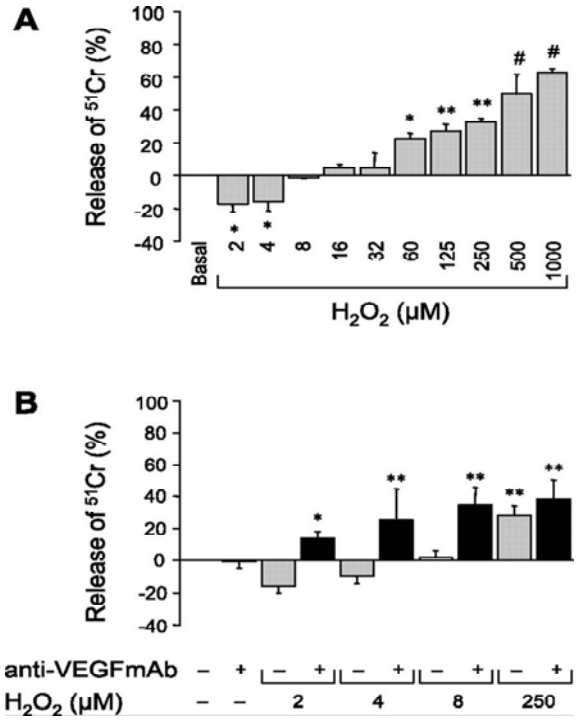

Figure 1. Cytotoxicity of H_2O_2: (A) ECs were treated with different concentrations of H_2O_2 (30 min) and cytotoxicity was examined 24 hr later. (B) ECs were treated with or without VEGFR2 blockade to demonstrate necessity of VEGFR2 in cell survival under different H_2O_2 concentrations.

Gonzalez-Pacheco et al., 2006

VEGFR2 and Redox Signaling

H_2O_2 has also been observed to be rapidly generated in response to VEGF binding to VEGFR2[143]. This oxidative burst requires PI3K activity and Ras-related C3 botulinum toxin substrate 1 (Rac-1) involvement. Rac-1 is a GTPase that is activated by PI3K. Once activated Rac-1 is a critical activator of NADPH oxidase and O_2^- generation[144].

Protein tyrosine nitration is a covalent modification in which a nitro (NO_2) group is added to a carbon of the aromatic ring of tyrosine[145]. This modification can alter protein function as well. It appears that tyrosine nitration can both inhibit and stimulate cell signaling[146-149]. Nitric oxide and O_2^- react to form $ONOO^-$. This reaction is highly favorable, in fact more so than the decomposition of O_2^- by SOD[150]. $ONOO^-$ is membrane permeable and has the ability to diffuse and influence processes up to two cells away[151]. $ONOO^-$ is a strong oxidant and can trigger the nitration of tyrosines in proteins. If oxidative damage from free radicals such as $ONOO^-$ detrimentally mediate cellular pathways the result is the perpetuation of chronic diseases.

El-Remessy et al. observed very interesting interactions of $ONOO^-$ and other oxidants with VEGFR2-mediated signaling in HUVECs. First, they observed an immediate increase in O_2^- , NO, and $ONOO^-$ in response to VEGF activation of VEGFR2. To determine the role of $ONOO^-$ in VEGF-mediated signaling, a specific $ONOO^-$ decomposition catalyst chemical was added; 5,10,15,20-tetraphenyl-21H, 23H porphine iron III chloride (FeTPPs). FeTPPs blocked the action of VEGF similar to results seen with polyethylene glycol - superoxide dismutase (PEG-SOD) and NG-nitro-L-arginine methyl ester (L-NAME). They also suggest that there is a feed forward

mechanism involving $ONOO^-$ to sustain VEGF signaling, and this action is due to oxidation and not nitration.

Oxidizing conditions appear to prime VEGFR2 for activation as observed by Oshikawa et al. Recently, these investigators used adenovirus transduction to anchor extracellular SOD into the heparin binding domain of ECs. Here, H_2O_2 generated extracellularly by SOD enhanced VEGF-induced VEGFR2 autophosphorylation in caveole, but not in other lipid rafts[152].

H_2O_2 and eNOS

Since H_2O_2 has the ability to critically affect endothelial dysfunction, its effect on eNOS has been heavily investigated. Drummond et al. saw a concentration dependent increase in eNOS protein in response to a 50 to 150 uM stimulus of H_2O_2 for 24 hr[153]. This increase in transcriptional activity was later determined to be mediated by a H_2O_2-induced inhibition of the Sp1 transcription factor[154]. Also, Cai et al. observed another redox sensitive pathway of eNOS transcription. H_2O_2 (100 uM for 1hr) also increased the expression of eNOS via Ca^{2+}/calmodulin-depedent protein kinase II (CaM kinase II) and janus kinase-2 (JAK2)[155]. Lauer et al. used transgenic mice over-expressing catalase to examine whether endogenously produced H_2O_2 would contribute to the up-regulation of eNOS expression caused by exercise training[156]. Their data strongly support the concept that H_2O_2 is critically involved in the up-regulation of eNOS by exercise. In transgenic mice over expressing catalase, and therefore exhibiting lower levels of H_2O_2, there was no significant increase in eNOS expression compared to wild-type littermates.

To further elucidate the role H_2O_2 has in what appears to be a juxtaposition of secondary messenger under physiological conditions, yet detrimental oxidant under pathological conditions, Hu et al. used H_2O_2 to examine the time course of eNOS activity in bovine aortic endothelial cells (BAECs). ECs were stimulated for 30 min, 2 hr, 4 hr, and 8 hr with 50, 200, and 500 uM of H_2O_2 in a time and dose dependent experiment (Figure 2). In further analysis using 500 uM, they observed an initial peak of eNOS activity at 30 min of exposure followed by decreasing levels of peNOS(1179) at 2, 4, and 8 hr[157]. pAkt(473) also followed a similar pattern.

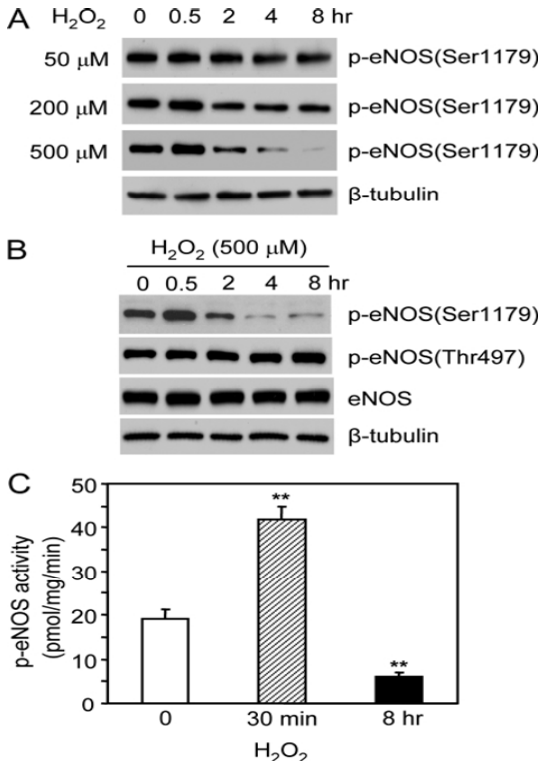

Figure 2. H_2O_2 Decreases eNOS Activity: (A) In bovine aortic endothelial cells (BAECs) different concentrations of H_2O_2were used over different lengths of time to examine eNOS activity. (B) & (C) 500 uM H_2O_2 was used to demonstrate a biphasic effect of H_2O_2 on eNOS activity.

Hu et al., 2008

Another interesting model of oxidative stress was developed by Boulden et al.[158]. This study used two repeated exposures to H_2O_2 (50 uM) for 30 min and found that after an initial (10 min) increase in NO production during the first exposure, by the end of the first 30 min NO production decreased. After changing media and applying another dose of H_2O_2, they observed the initial increase in NO production was significantly smaller than the first exposure. This model of endothelial dysfunction demonstrated diminished NO production which was ameliorated by BH_4 supplementation.

It appears that H_2O_2 is a potent secondary messenger for eNOS transcription, however activity of eNOS may be attenuated by further H_2O_2-induced oxidative stress. H_2O_2 has detrimental cellular affects under chronic exposure to heightened levels. *In vivo*, chronically elevated plasma levels of H_2O_2 seen in hypertensives may demonstrate similar cellular effects. Therefore, this study used a physiologically relevant 10 uM concentration of H_2O_2 along with increasing concentrations for 10min of exposure. As demonstrated by Gonzalez-Pachero et al., 30 min of H_2O_2 levels above 8 uM began to elicit cytotoxic results which were VEGFR2 dependent. In addition, Deskur et al. found *in vivo* levels of stage-3 hypertensives to range from 5.8 uM to 9.1 uM. The study examined the activity of eNOS following physiological H_2O_2 exposure coupled with VEGF stimulation.

Oxidant Production

In the Fenton reaction, free iron (Fe^{2+}) can be oxidized by H_2O_2 to ferric iron (Fe^{3+}) resulting in OH^- and $OH^\bullet$ formation. The production of $OH^\bullet$ in this Fenton reaction

is very reactive. Though this radical is short lived, it has the ability to damage virtually all types of macromolecules.

It has been observed that H_2O_2 enhances extracellular iron uptake in ECs via a transferrin receptor [159]. The intracellular iron pool also increases in response to H_2O_2 stimulation[160]. Tampo et al. observed that while H_2O_2 did increase eNOS activity, H_2O_2 impaired NO bioavailability. These observations point to another direction in which oxidative stress can impair NO bioavailability. However, the authors did not suggest any impairment of NO bioactivity via the Fenton reaction.

NADPH Oxidase Activation

ECs treated with H_2O_2 have demonstrated increased O_2^- production. There are various sources of O_2^- generation intracellularly. NADPH oxidase, xanthine oxidase, mitochondrial respiration, and uncoupled eNOS have all been proposed[161]. Coyle et al. stimulated porcine aortic endothelial cells (PAECs) with 60 uM H_2O_2 for 1.5 hr and examined the change in O_2^- levels. They observed an increase in intracellular levels of O_2^-, and this increase led to a cytotoxic response with time and dose dependency when they accessed H_2O_2-induced oxidative stress[162]. Using apocynin to inhibit NADPH oxidase Coyle et al. observed significant decreases in H_2O_2-induced O_2^- levels. Thus, NADPH oxidase is involved in H_2O_2-induced oxidative stress. It may be a mechanism for chronic H_2O_2 stimulation such as is observed in hypertensives for sustained production of O_2^- in endothelial dysfunction. In addition, H_2O_2 is also capable of increasing expression of p22phox, a subunit of NADPH oxidase, as Djordjevic et al. observed when hybrid ECs were stimulated with 10 and 50 uM of H_2O_2 for 3 hr[163].

Further, Witting et al. used PAEC to show a H_2O_2-induced increase in cellular O_2^- production and a decrease in Ach-induced NO accumulation. They also demonstrated that both processes were reversed by PEG-SOD, implicating O_2^- generation from H_2O_2 stimulus[164]. In addition, diphenyliodonium (DPI) and allopurinol inhibited NADPH oxidase and xanthine oxidase respectively which reduced H_2O_2-induced O_2^- generation.

eNOS Uncoupling

With respect to O_2^- generation through eNOS uncoupling, Coyle et al. used L-NAME to inhibit eNOS activity, this group determined that uncoupled eNOS also plays a role in the H_2O_2-induced increase in O_2^- levels in ECs. They subsequently used L-sepiapterin, which serves as a substrate for BH_4, to elucidate the mechanism of H_2O_2-induced uncoupling. It appears that H_2O_2 promotes oxidation of BH_4 as L-sepiapterin and apocynin completely returned H_2O_2-induced O_2^- generation back to control levels. The authors speculate that the H_2O_2-induced increase in NADPH oxidase produced O_2^- combines with NO produced from H_2O_2-induced increased eNOS activity to form $ONOO^-$ and thus oxidize BH_4[165].

Later, Witting et al. observed that H_2O_2 stimulated production of O_2^- was linked to endothelium-dependent vascular dysfunction, and they did not observed appreciable eNOS uncoupling. Their data indicated that eNOS did not contribute to cellular O_2^- generation[164].

Mitochondria

Mitochondria are significant sources of cellular ROS, and appear to be involved in H_2O_2-induced O_2^- generation. PAEC incubated with rotenone, an inhibitor of

mitochondrial respiration, significantly inhibited H_2O_2-induced O_2^- generation[166]. It also appears that mitochondria can sense and transduce a redox signal. Chen et al., used a 200 uM H_2O_2 exposure for 30 min on BAECs to demonstrate that H_2O_2-induced activation of epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and VEGF receptors were attenuated in cells deficient in respiring mitochondria[167]. Furthermore, the group found that the mechanism responsible for transduction of the redox signal was redox reactions. They found antioxidant supplementation diminished the H_2O_2-induced activation of receptors and downstream signaling.

Summary

VEGF is an important protein involved in endothelial function. It posesses potent vasoactive properties and maintining homeostatic levels are critical to long-term endothelial health. VEGF and NO appear to be inversely related *in vivo*, which may be due to oxidative stress. Therefore, identifying influences on VEGF-mediated eNOS activity under physiological stresses is essential. The benefits of exercise are established and mitigation of oxdiative stress is recognized as one of those benefits. However, a translational approach to understanding observed changes in human plasma biomarkers with alterations in signaling pathways *in vitro* is unique.

H_2O_2 is an oxidant that has been established as both an acute athero-protective molecule and a chronic atherogenic molecule. However, experimental conditions greatly influence these roles. It appears that H_2O_2 exposure over16 uM for 30 min induces cytotoxicity. However, many of the above studies used to at least 50uM H_2O_2 doses for 30+ min. Therefore, the direct effect of H_2O_2 that is below supra-pathological conditions

has not been studied in an endothelial dysfunction model. In addition, the VEGF-mediated activity of eNOS to non-pathological yet high physiological H_2O_2 levels has not been investiaged.

Finally, high phsyiolgoical levels of H_2O_2 are present in hypertensives, and atherogenic LSS levels are also associated with endothelial dysfunction. Therefore, to more accurrately characterize VEGF-mediated eNOS activity HUVECs will be pre-conditioned with an atherogenic level of LSS (4 dyne/cm^2) and athero-protective level of LSS (20 dyne/cm^2).

Statement of Purpose

The purpose of this research is to examine changes in VEGF-mediated eNOS activity under conditions of oxidative stress and homeostatis, both *in vivo* and *in vitro*. The oxidative stress reltationship involving plasma VEGF, NO, and H_2O_2 will be assessed in human participants both before and following 6 months of AEXT. *In vitro*, H_2O_2 exposure along with atherogenic and athero-protective levels of LSS will be used to characterize VEGF-mediated eNOS activity.

Specific Aims

1) Examine oxidative stress biomarkers in human subjects that demonstrate normotension, pre-hypertension, and HT before and following 6 months of AXET.

NO, VEGF, and H_2O_2 levels will be quantified in eligible subjects, before and after an AEXT intervention.

2) Demonstrate VEGF signaling cascades.

pAkt(473) and peNOS(1177) phosphorylation activity will be examine to establish the proposed model for retention of VEGF-induced signaling.

3) Determine VEGF-mediated eNOS activity in response to a H_2O_2 challenge in HUVECs.

peNOS(1177) activity will be examined in direct response to a physiological H_2O_2 level. Activity and phosphorylation of eNOS is necessary for VEGF-induced increases in NO. Therefore, decreases in VEGF-induced peNOS(1177) activity may be representative of detrimental effects of H_2O_2.

4) Determine activity of peNOS(1177) following atherogenic and athero-protective LSS pre-conditioning and VEGF stimulation.

The effect of 24hr LSS pre-conditioning on VEGF-mediated eNOS activity has not been examined previously. Therefore, application of LSS at 4 and 20 dyne/cm^2 will be used as an atherogenic and athero-protective stimulus, respectively. Pre-conditioning of this nature may influence VEGF signaling on peNOS(1177).

Hypotheses

1) It is hypothesized in a population of normotensive, pre-hypertensive, and hypertensive participants that lower NO levels will be related to higher BP levels. However, higher VEGF and H_2O_2 levels will be related to higher BP levels. Following AEXT, NO levels will increase while VEGF and H_2O_2 levels will decrease.

2) It is hypothesized that VEGF signaling will be preserved under all *in vitro* experimental conditions, however the activity level of pAkt(473) and peNOS(1177) will be reduced under certain conditions of the proposed model.

3) It is hypothesized that peNOS(1177) activity will be decreased following an oxidant challenge.

4) In addition, it is hypothesized that VEGF stimulation will have a greater effect on ECs following atherogenic LSS compared to athero-protective LSS.

CHAPTER 2

METHODS

Introduction

VEGF induces both an acute and sustained NO dependent relaxation[168-170]. It is a very important molecule in endothelial health[171-173] and therefore the ability of VEGF to activate eNOS is of critical importance. However, this activation has not been evaluated under physiological oxidant levels. H_2O_2 at a 10uM level would be considered a high circulating plasma H_2O_2 level *in vivo*[174-177]. This oxidant may affect the activity of eNOS in response to VEGF stimulation. Previous studies have shown that a 30 min exposure of ECs above this level induces cytotoxicity in a 24hr time period following exposure[178]. The dose response model used here takes into account the degradation of a bolus exogenous dose of H_2O_2. Therefore, VEGF-mediated eNOS activity under concurrent oxidizing conditions was observed. This was done by stimulating with VEGF and harvesting cells in a time course that allowed the oxidant to still be present.

Most oxidative stress studies on ECs have been done under static conditions, yet ECs *in vivo* are under constant exposure to biomechanical forces. Therefore, characterizing VEGF-mediated eNOS activity under an atherogenic and athero-protective environment *in vitro* may allow for more applicable insights into physiological signaling.

To further establish the role of VEGF in redox balance, plasma levels of VEGF, NO, and H_2O_2 were measured in participants. As described in the previous chapter,

increased circulating levels of VEGF may be a compensatory mechanism. Endothelial dysfunction with resulting HT may trigger such compensation, thus these biomarker levels were measured in normotensive, pre-hypertensive, and hypertensive participants before and after exercise training. Therefore, these measures were used to complement the *in vitro* model (Figure 3).

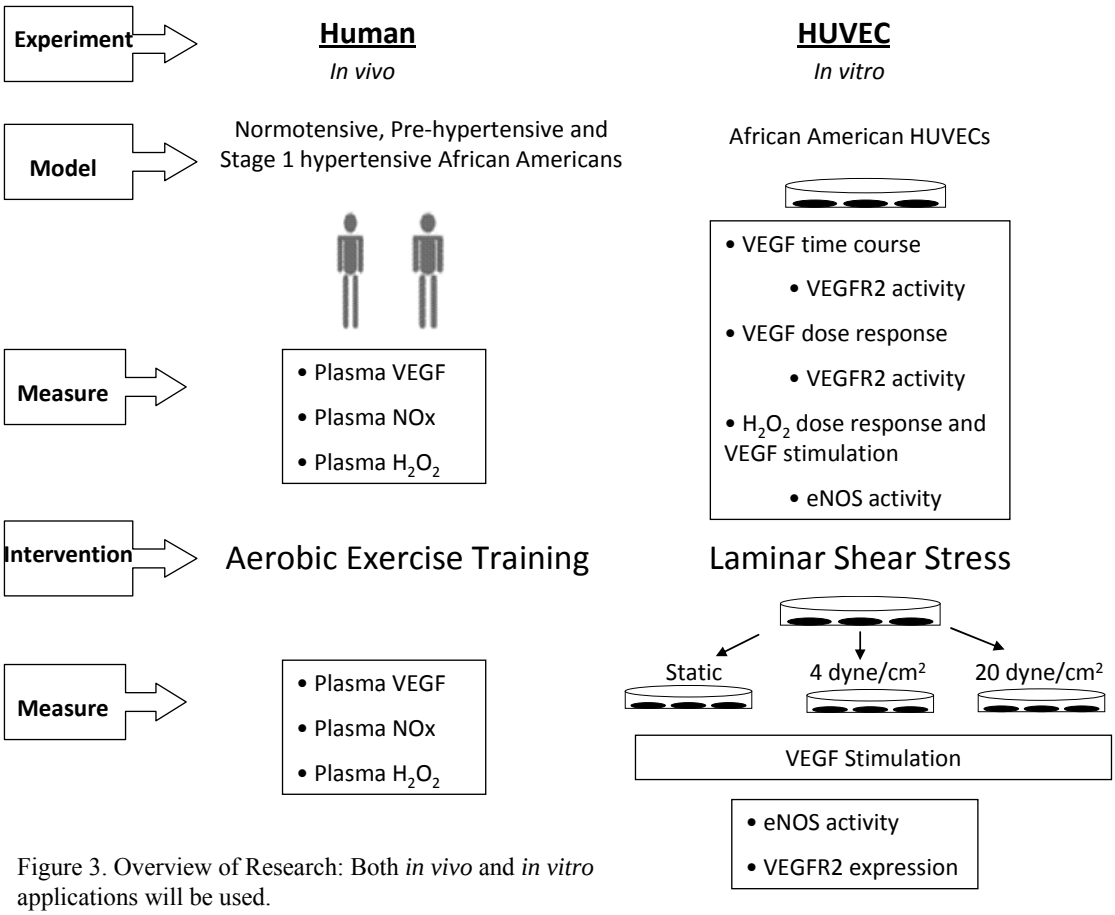

Figure 3. Overview of Research: Both *in vivo* and *in vitro* applications will be used.

Research Design

African American adults were recruited for entry into an exercise training intervention. Participants were screened for overt disease and educated on the American Heart Association's Step One diet. Baseline blood samples were then taken for later

analysis of VEGF, NO, and H_2O_2 biomarker levels. Baseline testing also included basic clinical exams such as body mass index (BMI), fitness capacity, BP measurement, and blood tests for cholesterol level, triglyceride level, and glucose level. Following 6 months of aerobic exercise training, participants repeated baseline testing.

HUVECs of African American ethnicity were used to optimize VEGF dosage, and length of exposure. In addition, H_2O_2 dose response was carried out with VEGF stimulation to assess eNOS activity. Next, HUVECs were exposed to atherogenic (4 dyne/cm^2) or athero-protective (20 dyne/cm^2) levels of LSS or kept under static conditions. Following 24hrs of LSS HUVECs were stimulated with VEGF and harvested. The activity of eNOS under these conditions in response to VEGF stimulation was examined. Cell lysate was also examined for VEGFR2 protein expression following LSS conditions.

In Vivo

Recruitment

African American men and women responding to media advertisement were contacted by telephone to assess their eligibility. Participants were sedentary (aerobic exercise < 2 times/week, < 20 min/session, sedentary job); 40 to 75 years of age; non-diabetic; non-smoking for at least one year; not on lipid lowering medication; suspected of having elevated BP (SBP >120 and <160 mmHg; DBP >80 and < 100 mmHg), and had no evidence of chronic obstructive lung disease, peripheral vascular disease, stroke, heart attack, lung disease, or heart disease. Participants also did not have any other medical conditions precluding vigorous exercise. Participants had a BMI < 40 kg/m^2.

Women on a hormone replacement therapy continued their therapy for the length of the study. Hypertensive participants using more than one antihypertensive medication were excluded.

Participants that met the criteria were scheduled for an orientation visit. In the orientation visit, participants had their medical history reviewed to determine if they had any criteria to exclude them from the study and the study was explained to them. Also, any questions were answered before participants provided written informed consent. This study was approved by the Temple University Institutional Review Board. After attending orientation, suitable participants were scheduled for screening.

Screening

At the first screening visit following an overnight fast, a blood sample was drawn for blood chemistries. Participants were excluded if they had fasting glucose >126 mg/dL, serum creatinine concentration >1.5 mg/dL, or total cholesterol >240 mg/dL. Height and weight were also measured to confirm BMI criteria (BMI < 40 kg/m^2). Qualified participants proceeded to the second screening visit. A physical and cardiovascular examination was administered by a study physician. Following a successful exam, participants were scheduled for an exercise stress echo test. The test was performed at Temple University Hospital with a cardiology fellow, echo technician, and a member of the Hypertension Molecular and Applied Physiology (HyMAP) lab who was trained in conducting exercise electrocardiograms (ECGs). The bicycle exercise stress test has three 3 min stages: 25, 50, and 75 W. An echocardiogram was taken at rest, 1:30 minutes into each stage, and in recovery. The participants were monitored by ECG

and BP was taken every 2 minutes. Participants had a <2 mV ST-segment depression and no cardiovascular signs/symptoms to be included in the study.

Under the supervision of the study physician, qualified participants who were using one anti-hypertensive medication were tapered off the medication and remained off for the remainder of the study. The recommended tapering schedule began at the start of the dietary stabilization period. All participants had their BP measured weekly by study personnel during the remainder of the study. If a participants' SBP or DBP was >159 or >99 mmHg, respectively, for three consecutive weeks at any time during the study, they were excluded from further participation and referred back to their private physician.

Dietary Stabilization

Qualified participants met once a week for six consecutive weeks with a study dietician and learned how to maintain the American Heart Association Step One diet. This low fat (total fat <27% of calories), low sodium (<2,300 mg) diet was maintained throughout the study. Three day diet recall logs were used by the nutritionist to monitor changes in dietary intake prior to and following class participation. In addition, participants were asked to complete subsequent diet logs at week 8, 16, and 24 of exercise training.

Testing

Following the orientation visit, screening visits, and dietary stabilization period, participants then progressed to testing. In order to characterize the participant demographic, testing consisted of assessment of body composition, BP level, submaximal exercise test, and a blood draw. Body composition was measured by bio-electrical

impedance (BIA). An ImpediMed® ImpDF50 body composition analysis device was used to assess fat mass and free fat mass. Participants performed a submaximal treadmill test to estimate their maximal oxygen consumption (VO_{2max}). Using a Viasys Vmax® Encore gas analysis system VO_2 was determined at given work rates of a modified Bruce protocol. Termination criteria was the attainment of 75 to 80% of heart rate reserve. Regression analysis using VO_2 values and HR values determined at each stage were used to estimate VO_{2max}.

BP levels were measured according to JNC VII guidelines. Casual BP levels were measured three times on three separate visits to the lab and averaged. Finally, following a 12hr overnight fast blood was drawn in EDTA tubes and spun at 16000 g at 4 °C for 20 min. Plasma was subsequently aliquoted and frozen at -80 °C until used. All colorimetric assays were read on a VERSAmax® spectrophotometer.

Exercise Intervention

Participants were required to undergo exercise training 3 days/week for 6 months. All sessions were supervised by study personnel. Participants were gradually progressed up to required duration and intensity. Initiation of exercise training began at 20 min per day, at an intensity of 50% predicted VO_{2max}. Five minutes of time was added each week of completed training until 40min was reached. Next, intensity was increased 5% each week following attainment of exercise duration at 40 min. Participants progressed up to 65% VO_{2max} and this intensity was maintained for the remained of the intervention.

Participants exercised aerobically by utilizing treadmills, stationary bicycles, elliptical machines, rowing ergometers, and stair steppers. Intensity was monitored via

HR monitors, and adherence to personalized HR prescriptions was checked every 10 min and recorded by study personnel. In addition, BP was measured before and following exercise. Weight changes were monitored with weekly weighing. Adherence was assessed by examining individual training logs for frequency data. Following completion of the exercise training intervention, baseline testing was repeated. A summary of the *in vivo* recruitment, screening, baseline testing, training, and final testing is presented in Figure 4.

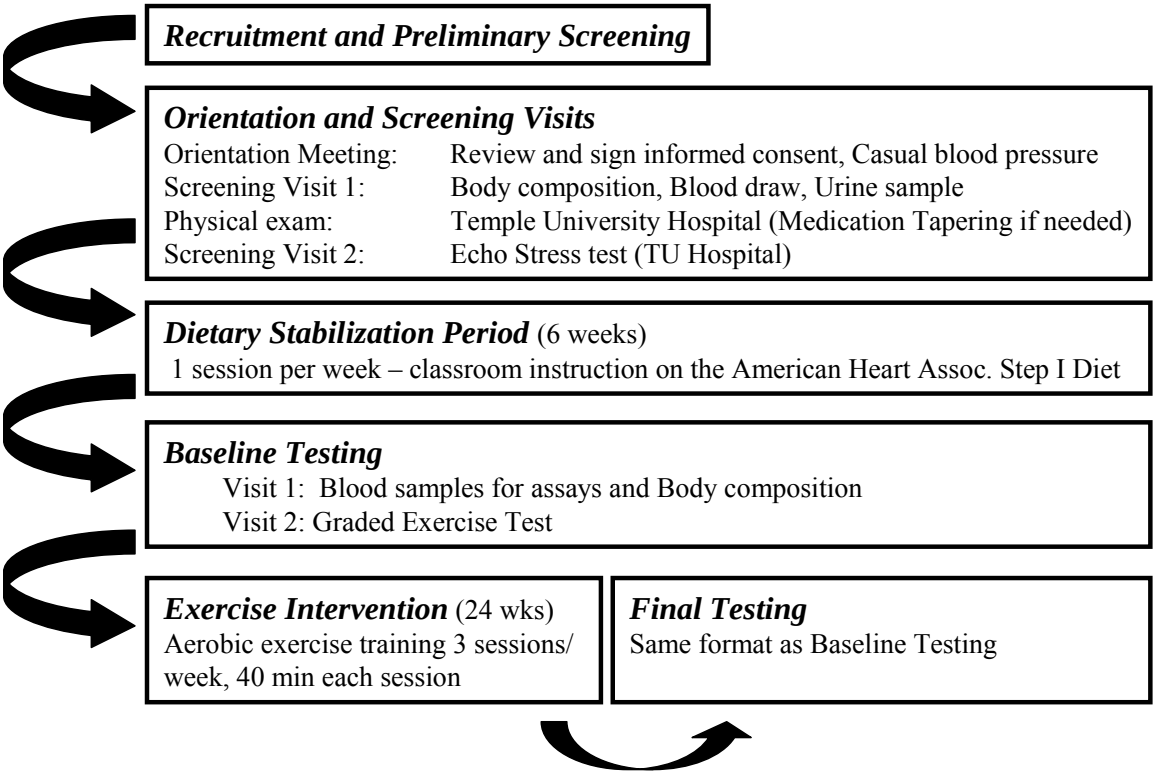

Figure 4. *In Vivo* Flow Chart: Participants were extensively screened and educated on nutritional intake prior to testing. Following completion of the exercise training intervention participants completed final testing.

VEGF Assay

An enzyme-linked immunosorbent assay (ELISA) from R&D Systems® was used to quantify human plasma VEGF levels. In this sandwich enzyme immunoassay, samples and standards were added to wells pre-coated with VEGF monoclonal antibody. After washing away unbound substances, a VEGF enzyme-linked polyclonal antibody conjugate was added. Excess antibody was washed and a substrate solution was added for color development. A stop solution was added to the wells and absorbance was read at 540 nm. The inter-assay coefficient of variation (CV) was 9.3% and the intra-assay CV 8.3%.

NO Assay

To determine NO levels, NO end products, nitrate and nitrite, were measured with an Enzo Life Sciences®/Assay Designs® assay kit. Plasma samples were diluted with reaction buffer and filtered through a 10,000 molecular weight cut off (MWCO) filter. In this assay, nitrate is converted to nitrite by the enzymatic process of nitrate reductase. Nitrite levels are then visualized following production of an azo dye via the Griess reaction. Absorbance was read at 540 nm. The inter-assay CV was 7.6% and the intra-assay CV 10.6%.

H_2O_2 Assay

Using an assay from Invitrogen®, H_2O_2 was quantified via Amplex® Red reagent. The chemical, (10-acetyl-3,7-dihydroxyphenoxazine), reacts with H_2O_2 in a 1:1 stoichiometric reaction to give a red oxidation product called resorufin which can be read at an absorbance of 560 nm. The inter-assay CV was 7.9% and the intra-assay CV 2.7%.

In vitro

Materials

African American HUVECs were acquired from LONZA Clonetics® and cultured at 37°C and 5% CO_2 tension. All media was acquired from LONZA. Endothelial Growth Media (EGM) contains basal media supplemented with 2 ml bovine brain extract, 0.5ml human endothelial growth factor, 0.5 ml hydrocortisone, 0.5 ml gentamicin/amphotericin B (GA)-1000, 10 ml fetal bovine serum (2%). Cells were grown until 90% confluent prior to any stimulation.

LSS was conducted via cone and plate viscometer under above culturing conditions. H_2O_2 (30%) was obtained from Sigma-Aldrich®. Human recombinant VEGF was obtained from Cell Signaling Technology®. Antibodies for: Akt, pAkt(473), VEGFR2, and phosphorylated VEGFR2 – tyrosine 1175 (pVEGFR2(1175), were obtained from Cell Signaling Technology®, eNOS, and peNOS(1177), were obtained from BD Transduction Laboratories™. Finally, anti-actin was obtained from Sigma-Aldrich®. Mouse and Rabbit secondary antibodies were obtained from Jackson ImmunoResearch Laboratories Inc®.

Immunoblotting

Immunoblotting was performed on protein lysate obtained under experimental conditions. HUVECs were harvested in radio-immuno precipitation assay (RIPA) buffer or NP-40 lysis buffer. Samples were then centrifuged at 15000 g at 4 °C for 20 min and supernatant was collected. Protein concentration was determined via Bradford (for RIPA harvests) or Pierce® 660 nm Protein (for NP-40 harvests) assay using a VERSAmax®

spectrophotometer set at 595 nm or 660 nm respectively. Following dilution with 5X sodium dodecyl sulfate (SDS) and boiling of protein samples, 10% acrylamide gels were used to separate loaded protein by molecular weight. Immunobilon polyvinylidene fluoride (PVDF) membrane (Millipore®) was used for immunoblot transfer. Tris buffered saline with .05% tween (TBST) supplemented with appropriate agent, 5% bovine serum albumin (BSA) or 5% non-fat dried milk (NFDM), was used for blocking. Blots were incubated with primary antibody at 4 °C overnight. Following washes in TBST, appropriate secondary antibody was added for 1 hr incubation. The blot was then washed with TBST and followed with a final wash in TBS. Enhanced chemi-luminescence (ECL) solution was then used to image protein banding. Chemi-luminescence was detected with an Alpha Innotech® FluorChem FC2 camera.

Statistical Analysis

Immunoblot images were characterized using the National Institute of Health's ImageJ program. Student's t-test was used to test for differences in protein level between experimental conditions. All western blot analyses were actin adjusted. Changes in pVEGFR2 to VEGFR2 ratio were examined under static conditions following VEGF activation to examine time and dose responses. peNOS to eNOS ratio following H_2O_2 exposure and VEGF activation, and also under LSS conditions with VEGF activation was examined. *In vitro* analysis also included assessment of VEGFR2 expression under different flow conditions. *In vivo*, relationships between normotensive, pre-hypertensive, and hypertensive groups, and VEGF, H_2O_2, and NO levels were evaluated with ANOVA. In addition, the correlation between these biomarkers was also explored; VEGF*H_2O_2,

VEGF*NO, and H_2O_2*NO. Data are presented as mean ± SEM, and α level was set at .05 for analysis.

Limitations

While this *in vitro* model used physiological levels of H_2O_2 and LSS, it is understood that application of H_2O_2 under static conditions was not a true mimetic of *in vivo* conditions. In addition, the cessation of LSS may increase ROS levels. However, the application of LSS for 24 hr is an established *in vitro* model of exercise. In addition, this model was used to create atherogenic and athero-protective conditions and observe the differences in VEGF signaling properties which may help elucidate how oxidative stress changes can influence ligand-induced signaling and outcome molecules seen *in vivo*. Therefore, this model does not allow for definitive clarification of mechanism involved in the differences observed under atherogenic and athero-protective conditions.

CHAPTER 3

RESULTS

Baseline Clinical Demographics and Biomarkers

To determine the *in vivo* interaction of VEGF, H_2O_2, and NO these biomarkers were measured in participants before the exercise training intervention. On average, participants did not demonstrate hypercholesterolemia, hypertriglyceridemia, hyperglycemia, HT, or kidney disease (Table 1). However, participants were obese, pre-hypertensive, and sedentary.

Table 1. Baseline Demographics

Variable	Baseline
Age (yrs)	51.8 ± .97
Male/Female	8 (17%) / 40 (83%)
Cholesterol (mg/dL)	188.2 ± 3.33
Triglycerides (mg/dL)	87.2 ± 5.98
HDL (mg/dL)	65.2 ± 3.03
LDL (mg/dL)	105.5 ± 3.38
Glucose (mg/dL)	94.6 ± 1.38
SBP (mmHg)	124.9 ± 1.78
DBP (mmHg)	79.5 ± 1.08
BMI (kg/m^2)	32.2 ± 0.82
VO_{2max} (ml/kg/min)	27.0 ± 0.81
GFR (ml/min)	95.3 ± 2.37

African American participants without overt disease enrolled in the study. Mean ± SEM, N=48.

Baseline measures of plasma biomarkers indicated trends associated with BP category. VEGF levels and NO levels demonstrated inversely related trends (Figure 5). VEGF levels increased with increasing BP level while NO levels decreased from normotensive to hypertensive participants. H_2O_2 levels also trended upward with increasing BP level (Figure 6). Correlation analysis did not reveal any significant association between plasma biomarkers (VEGF*H_2O_2, p=.3; VEGF*NO, p=.9; H_2O_2*NO, p=.3).

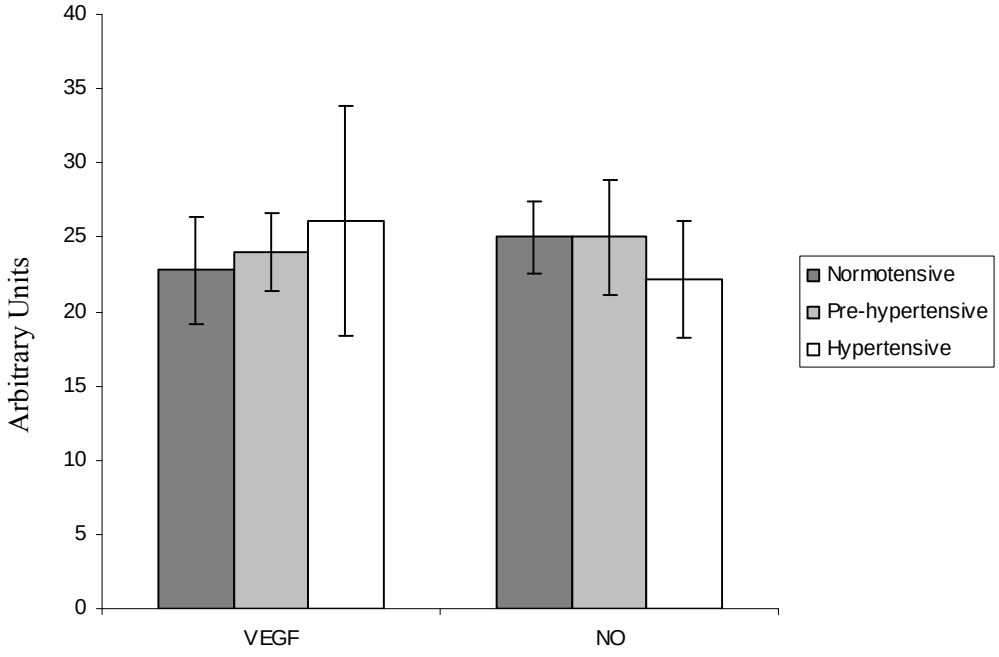

Figure 5. Baseline VEGF and NO Biomarkers: Average plasma VEGF (pg/ml) and NO (µmol/L) levels for normotensive (n=17), pre-hypertensive (n=22), and hypertensive (n=9) African Americans at baseline are displayed. Mean ± SEM, N=48.

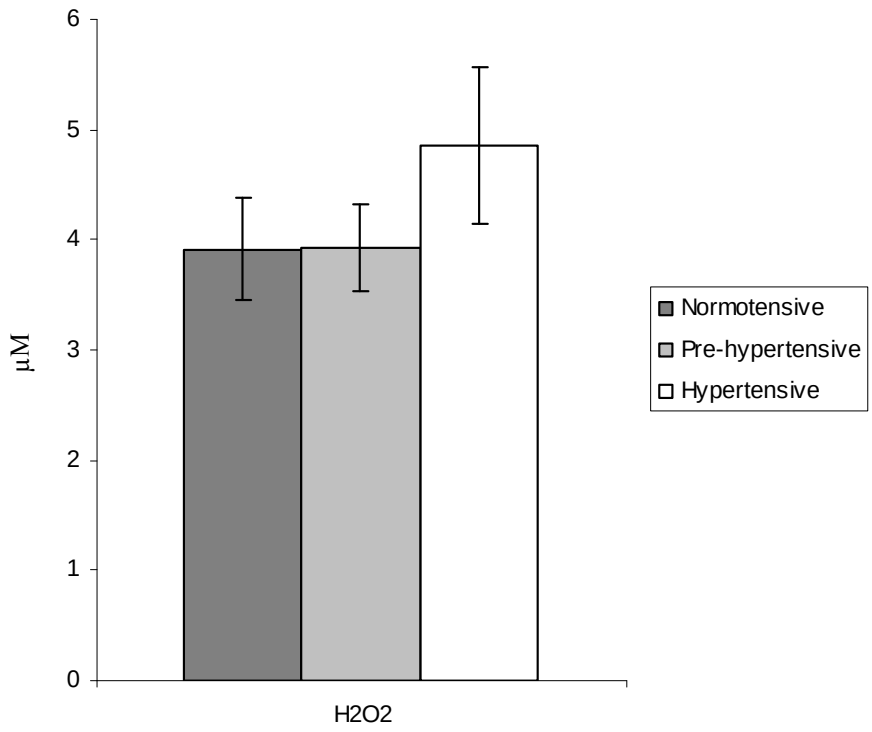

Figure 6. Baseline Plasma H_2O_2 Level: Average plasma H_2O_2 levels for normotensive (n=17), pre-hypertensive (n=22), and hypertensive (n=9) African Americans at baseline are displayed. Mean ± SEM, N=48.

Change in Clinical Measures and Biomarkers Following Exercise Training

Twenty-two participants completed the 6 month exercise training intervention. Several subjects were not able to complete several tests resulting in variation in the samples sizes for the final clinical variables (Table 2). However, a significant decrease in triglycerides was observed along with a significant increase in fitness capacity as indicated by VO_{2max}. VEGF levels did not significantly change following 6 months of exercise training, yet NO levels did increase significantly (Figure 7). H_2O_2 levels did not change with AEXT (Figure 8).

Table 2. Change in Clinical Variables Following 6 Months of AEXT

Variable	Change
Cholesterol (mg/dL)	-6.6 ± 9.08 (n=19)
Triglycerides (mg/dL)	-12.9 ± 6.18 (n=19) *
HDL (mg/dL)	-0.1 ± 4.40 (n=19)
LDL (mg/dL)	-3.1 ± 7.02 (n=19)
Glucose (mg/dL)	-5.4 ± 3.80 (n=17)
SBP (mmHg)	1.9 ± 1.91 (n=22)
DBP (mmHg)	1.6 ± 1.39 (n=22)
BMI (kg/m^2)	-0.5 ± 1.28 (n=22)
VO_{2max} (ml/kg/min)	4.0 ± 1.51 (n=22) *

African American participants completed 6 months of AEXT, 3 days per week for 40 min. Mean ± SEM, * $p<.05$ between baseline and final values.

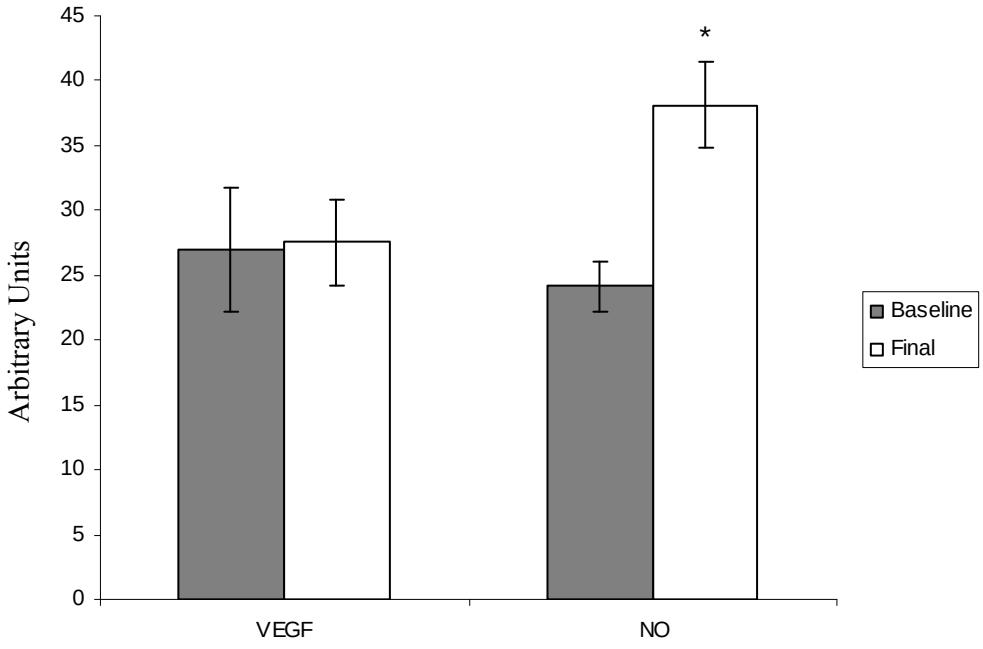

Figure 7. Change in VEGF and NO Biomarkers: Average plasma VEGF (pg/ml) and NO (μmol/L) levels at baseline (dark gray) and final (white) are displayed. Mean ± SEM, N=22, *$p<.05$ between baseline and final.

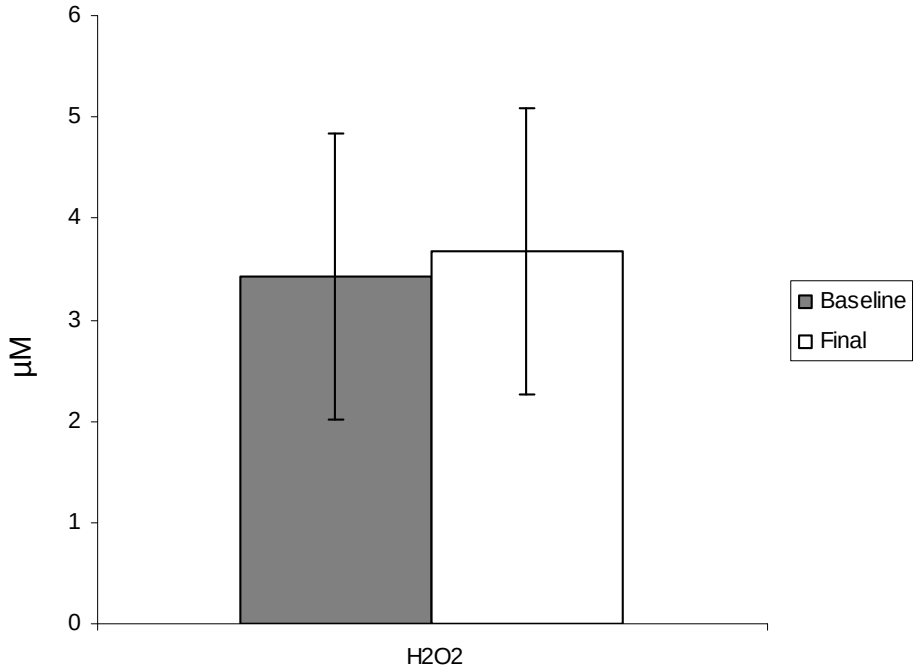

Figure 8. Change in Plasma H_2O_2 Level: Average plasma H_2O_2 levels at baseline (dark gray) and final (white) are displayed. Mean ± SEM, N=22.

Given that there was not and overall change in BP following AEXT, the differences between participants that responded to AEXT with a decrease in BP and those participants that responded with an increase in BP were investigated. To determine whether plasma biomarkers changed relative to BP changes, participants were divided into groups relative to their BP change following the intervention. When the group was divided by BP response to AEXT, it allows us to uncover potential alleviation of compensatory mechanisms in those participants that experienced a beneficial drop in BP level following AEXT.

Participants that decreased their SBP or DBP also demonstrated a decrease in VEGF level. This difference of VEGF levels between groups of participants based on

their response to AEXT approached significance (p=.06). In addition, an increase in NO level was observed as well in both groups following AEXT regardless of BP response (Figure 9).

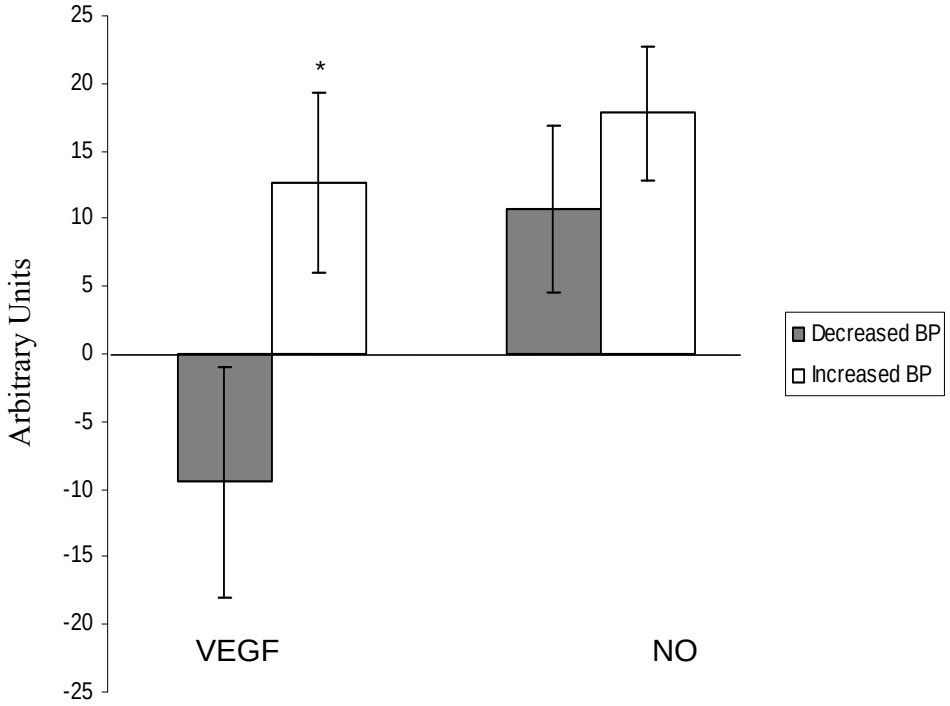

Figure 9. BP Changes with VEGF and NO Biomarkers Following AEXT: Participants were grouped according to BP change. A decrease in either SBP or DBP following 6 months of AEXT is presented by dark gray bars (N=12). An increase in both SBP and DBP is presented in the white bars (N=10). Mean ± SEM, *p<.06 between groups.

Participants with an increase in BP following 6 months of exercise training displayed a slightly increased level of H_2O_2 (Figure 10). Thus, participants with an increase in BP level following 6 months of exercise training displayed an increased VEGF level, increased H_2O_2 level, and increased NO level. While participants that lowered their BP level following AEXT displayed decreased VEGF levels, did not change H_2O_2 levels, and increased NO levels.

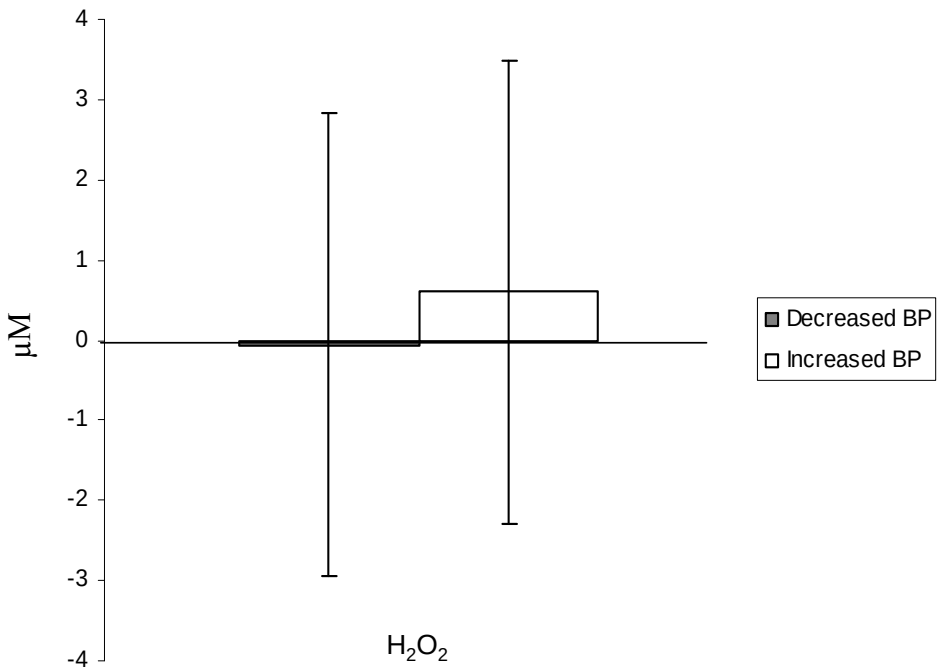

Figure 10. BP Changes with Plasma H_2O_2 Following AEXT: Participants were grouped according to BP change. A decrease in either SBP or DBP following 6 months of AEXT is presented by dark gray bars (N=12). An increase in both SBP and DBP is presented in the white bars (N=10). Mean ± SEM.

These results suggest that while exercise training is beneficial in some participants for lowering BP, in participants without a decrease in BP there may be compensatory mechanisms still in place. Comparatively elevated VEGF levels may be a factor in the likewise comparatively elevated NO levels, however the presence of ROS such as H_2O_2 may continue to shift the redox balance and thus prevent ultimate phenotypic changes in BP following an exercise intervention. Using *in vitro* models, this possible interaction was investigated further to elucidate a vascular mechanism of regulation.

VEGF Stimulation Optimization

VEGF levels are elevated in hypertensives[179;180], and there appears to be an increasing level of circulating VEGF according to BP category in the present study (Figure 5). Therefore, investigating the effects of VEGF signaling on vascular mechanisms is important in understanding the progression of HT. In this experiment, the optimal concentration of VEGF was determined. *In vivo*, circulating levels of VEGF are in the pg/ml range[181-183]. It is important to note that, *in vitro*, ng/ml concentrations are commonly used for stimulation[184;185]. It was observed that the pg/ml level of VEGF did not elicit a detectable signal, and that pVEGFR2 reached maximal stimulation at 50 ng/ml of VEGF for 10 min (Figure 11).

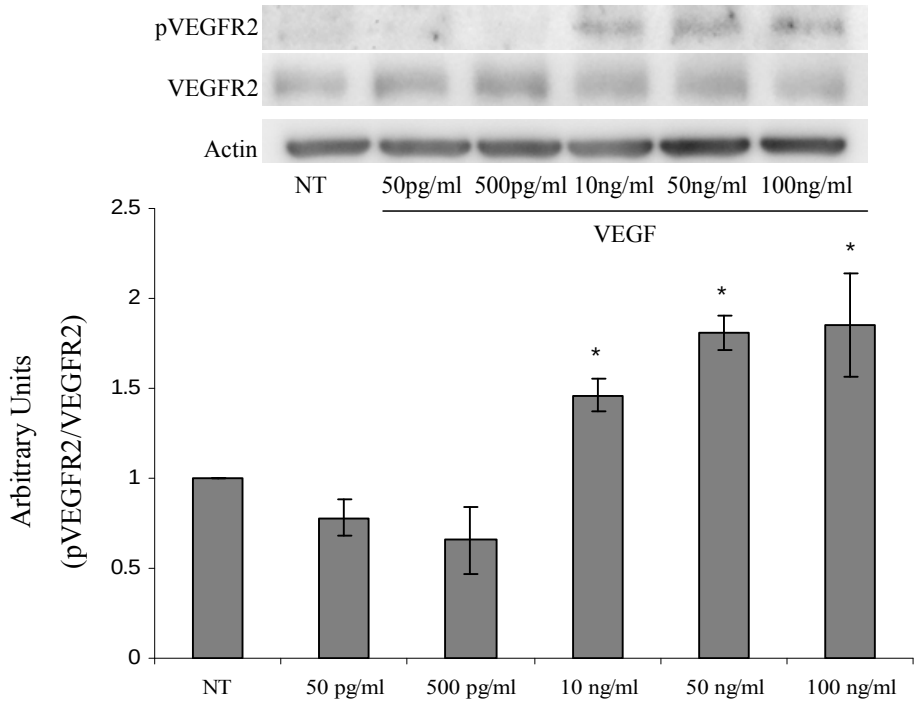

Figure 11. VEGF Dose Response: Representative western blot and quantification showing the relative amounts of pVEGFR2 protein versus VEGFR2 protein in HUVECs following a 10 min stimulation with indicated amount of VEGF. Mean ± SEM, N=3, *p<.05 from NT.

It has been previously reported that ligand stimulated VEGFR2 auto-phosphorylation is a kinetic event[186]. To determine the length of VEGF stimulation for optimal detection of pVEGFR2, a time course experiment was carried out. A 2 to10min window of stimulation was sufficient to significantly increase pVEGFR2 activation (Figure 12). As 2 min of VEGF stimulation significantly increased VEGFR2 activity, this duration was used for all subsequent VEGF stimulation experiments.

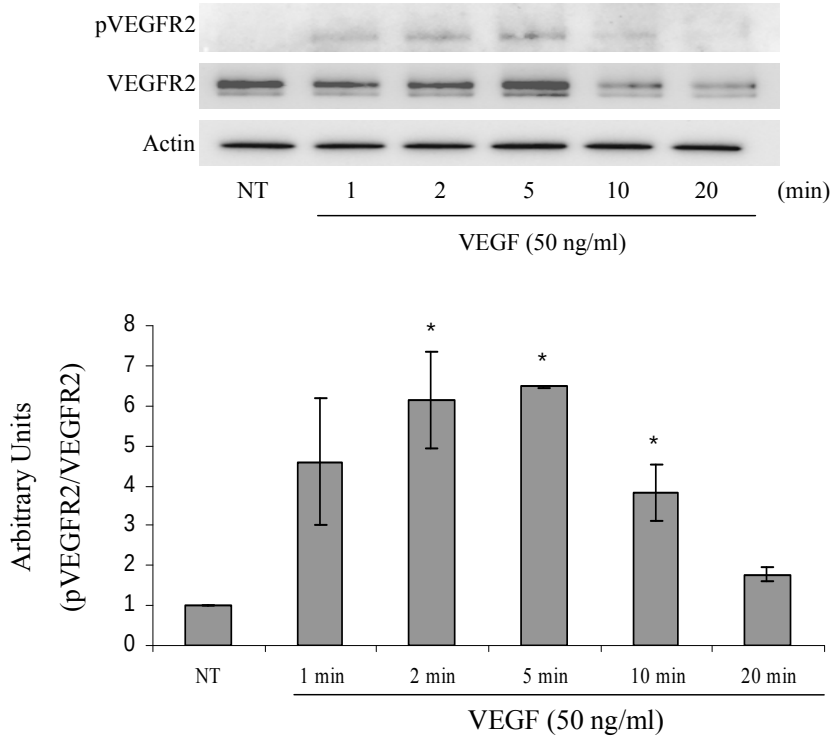

Figure 12. VEGF Time Course: Representative western blot and quantification showing the relative amounts of pVEGFR2 protein versus VEGFR2 protein in HUVECs following VEGF stimulation for the indicated duration. Mean ± SEM, N=3, *p<.05 from NT.

H_2O_2 Effect on VEGF Stimulated eNOS Activity

To investigate the role of H_2O_2 on VEGF stimulated eNOS activity a dose response experiment was carried out. H_2O_2 exposure duration was 10 min, followed by 2

min VEGF stimulation. The 10 min time point was chosen due to the rate of degradation of H_2O_2 in media (Figure 13). It was observed that exogenous H_2O_2 in cell culture media degrades rapidly, and that 30 min following addition of the lowest dose (10 uM H_2O_2) no detectable H_2O_2 levels remained. At 10 min, H_2O_2 is still present in the cell culture media and therefore the direct effects of the oxidant are active. This more closely resembles an *in vivo* situation as circulating plasma is always in contact with the endothelium.

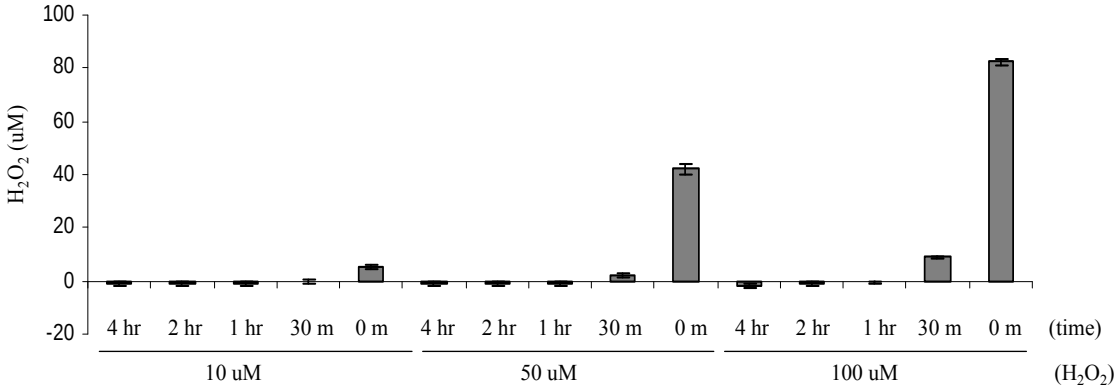

Figure 13. H_2O_2 Degradation in Culture Media: EGM media aliquots were spiked to final H_2O_2 concentrations of 10 uM, 50 uM, and 100 uM. Samples sat at RT for indicated times (4 hr, 2 hr, 1 hr, 30 min, Immediate) prior to assay. Mean ± SEM, N=3

VEGF stimulation significantly increased peNOS levels under 0uM H_2O_2 and application of a physiologically relevant 10 uM H_2O_2 still maintained significant VEGF stimulated peNOS levels (Figure 14). Under higher levels of H_2O_2, 50 uM and 100 uM, VEGF stimulation was no longer significantly increased over basal levels. This decrease of VEGF mediated eNOS activity is displayed in Figure 15.

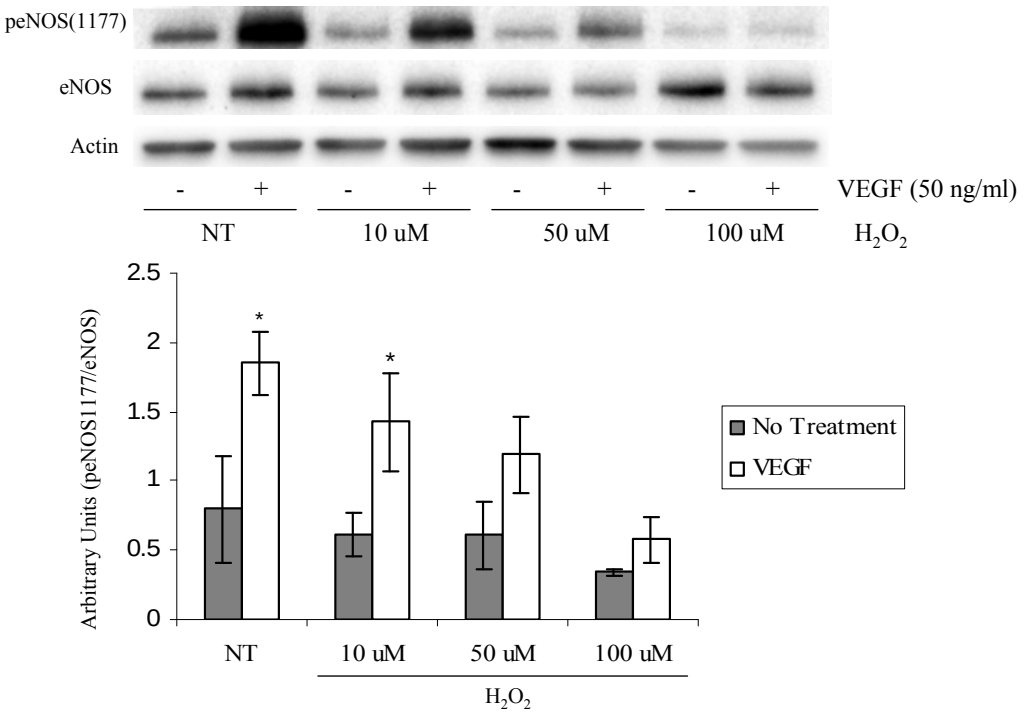

Figure 14. H_2O_2 Dose Response and VEGF Stimulation – eNOS: Representative western blot and quantification showing the relative amounts of peNOS protein versus eNOS protein in HUVECs following H_2O_2 exposure for 10 min (dark bars), followed by VEGF stimulation for 2 min (white bars). Mean ± SEM, N=3, *p<.05 from condition's no treatment control.

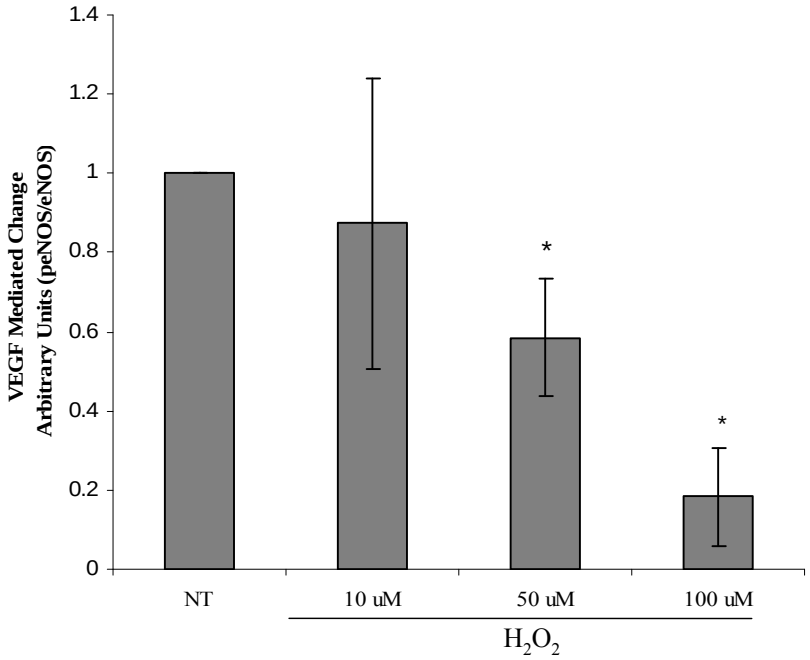

Figure 15. H_2O_2 Dose Response – VEGF Mediated eNOS Activity: Graph displays peNOS/eNOS difference between no treatment controls and VEGF stimulation following H_2O_2 exposure. Mean ± SEM, N=3, *p<.05 from NT.

Akt activity was also assessed under these conditions (Figure 16). There was no significant change in Akt activity under any condition. The experimental design did not include a serum starvation period and previous reports indicate that serum independently activates pAkt(473)[187-189]. This phosphorylation site may be saturated under the current conditions thus not allowing for observable changes in activity from H_2O_2 or VEGF.

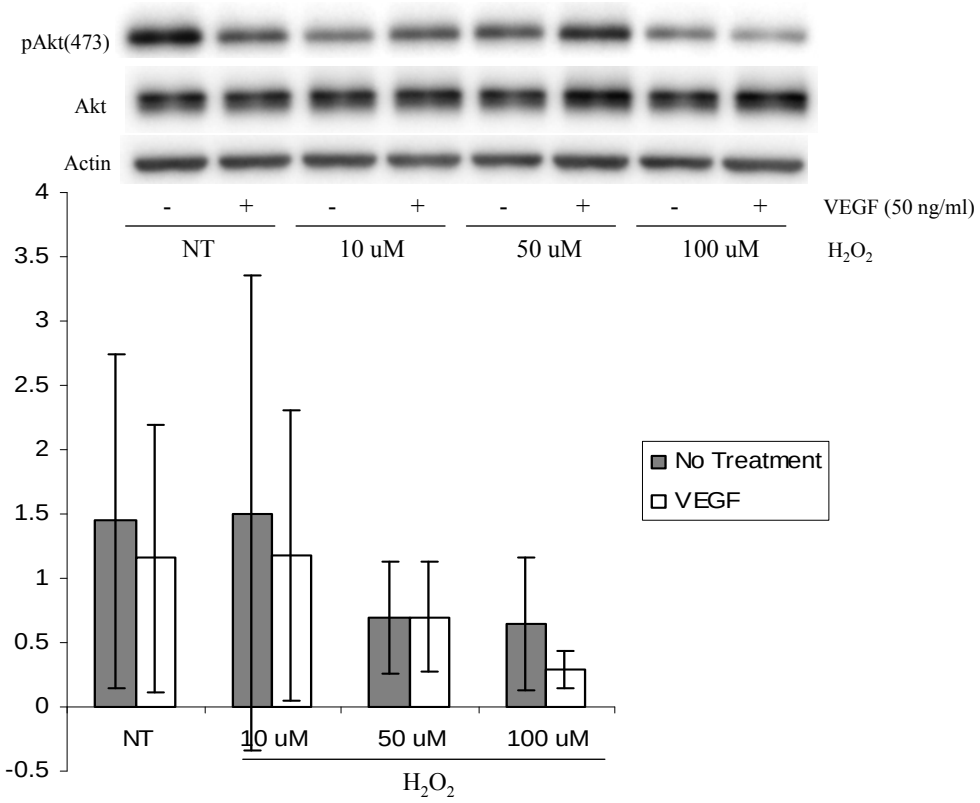

Figure 16. H_2O_2 Dose Response and VEGF Stimulation – Akt: Representative western blot and quantification showing the relative amounts of pAkt protein versus Akt protein in HUVECs following H_2O_2 exposure for 10 min (dark bars), followed by VEGF stimulation for 2 min (white bars). Mean ± SEM, N=3.

Laminar Shear Stress Experiments

The above results suggest that H_2O_2, a ROS, is detrimental to VEGF mediated eNOS activity, therefore, we investigated the role of atherogenic (4 dyne/cm^2) and athero-protective (20 dyne/cm^2) levels of LSS for 24 hr on VEGF mediated signaling.

Following 24 hr of LSS at 20 dyne/cm^2, VEGF mediated eNOS activity was significantly less than VEGF-mediated eNOS activity after 24 hr of LSS at 4 dyne/cm^2 (Figure 17).

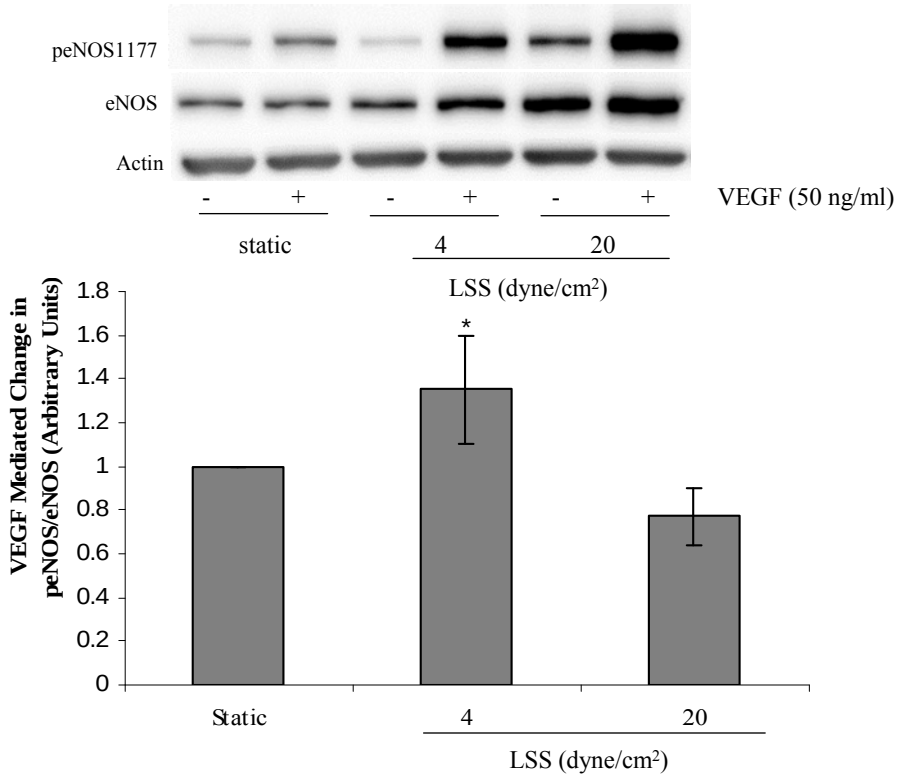

Figure 17. VEGF-Mediated Change in eNOS Activity Following 24 hrs LSS: Representative western blot and quantification showing the relative amounts of peNOS1177 to eNOS protein in HUVECs that underwent 24 hrs of LSS at atherogenic or athero-protective magnitudes followed by 2 min of VEGF stimulation. Mean ± SEM, N=3 * p>.05 between 4 and 20 dyne/cm^2

In order to clarify a possible mechanism for this difference in VEGF mediated eNOS activity, VEGFR2 density was examined. A significant difference was observed between VEGFR2 expression levels following 24 hr LSS (Figure 18). There was significantly less VEGFR2 protein under 20 dyne/cm^2 LSS relative to 4 dyne/cm^2 LSS and static conditions.

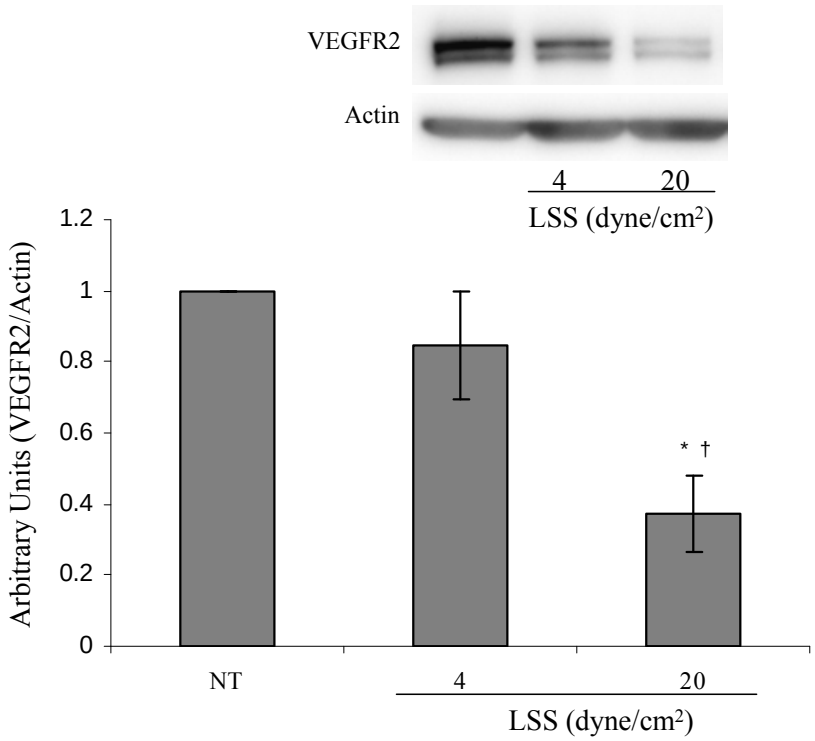

Figure 18. VEGFR2 Expression Following 24 hr LSS: Representative western blot and quantification showing the relative amounts of VEGFR2 protein versus actin protein in HUVECs following 24 hr LSS. Mean ± SEM, N=3, *p<.05 from NT, † p<.05 from 4 dyne/cm^2.

CHAPTER 4

DISCUSSION

Introduction

Oxidative stress is a contributing factor in endothelial dysfunction and endothelial dysfunction is a key component in the pathophysiology of CVD. Maintaining eNOS activity is critical for NO dependent relaxation of the vasculature. Chronic imbalance of cellular redox status impairs endothelial function. There are many proteins and events that activate eNOS. One such protein is VEGF. VEGF has been implicated in NO production for angiogenesis[190;191], proliferation[192], and vasoactive functions[193;194]. Thus, clarifying influences on VEGF-mediated signaling under physiological stresses is essential.

In Vivo – Compensatory Feedback Loop

Oxidative stress biomarkers were examined in human participants that demonstrated normotension, pre-hypertension, and HT. As previously discussed, several studies have investigated these biomarkers individually in participants with advanced disease states[195-199]. This study is the first to investigate these biomarkers in a population of at-risk participants with sub-clinical disease status.

It was hypothesized that higher VEGF and H_2O_2 levels would be related to higher BP levels and lower NO levels would be related to higher BP levels. While not significant, the results confirm a trend toward the hypothesized association. VEGF and

H_2O_2 levels demonstrated an upward trend with increasing BP level in a population of African American adults without overt disease. In addition, NO levels were also decreased in hypertensives relative to pre-hypertensive and normotensive participants.

These findings suggest a compensatory increase in VEGF levels in the presence of oxidative stress as characterized by elevated H_2O_2 levels and decreased NO levels. When ECs are dysfunctional NO levels are diminished due to an increase in ROS such as H_2O_2. Thus, an increase in an eNOS activator would be beneficial. In hypertensives, chronic endothelial dysfunction is a pro-atherosclerotic environment. VEGF has many cellular functions and one is to increase NO production and eNOS expression[200-202]. Therefore, elevated levels of VEGF in hypertensives could be viewed as a compensatory mechanism as they have demonstrated a reciprocal regulation with NO as well[203].

Following 6 months of AEXT, NO significantly increased from baseline to post exercise training in the whole population. Previous studies have demonstrated the beneficial effects of AEXT [204-206]. However, this study utilizes AEXT as a preventative strategy to slow the progression of CVD in at-risk African Americans. Therefore, we also conducted a post-hoc analysis based on the change in BP level following AEXT. As 6 months of AEXT may greatly influence many systemic influences on BP regulation, it is possible that the adaptive responses in those participants that lowered their BP have alleviated the compensatory elevation of VEGF levels that we saw at baseline in hypertensives. In deed, VEGF does have several redox sensitive transcription factors.

It was observed that participants which demonstrated a decrease in BP following AEXT, VEGF levels also decreased. Participants that did not exhibit beneficial exercise-induced lowering of BP had an increase in VEGF levels that accompanied elevated H_2O_2 and NO levels. The characterization of these plasma biomarkers in the two groups may lend insight into the condition of the endothelium during the progression of CVD.

Systemic BP regulation has many facets, and exercise also affects many mechanisms to induce changes in BP. Perhaps those participants that lowered their BP levels with AEXT have returned to a homeostatic level of ROS. It may be that their redox balance changed from a chronic state of heightened oxidative stress to a functional level in which ROS can now properly function as secondary messengers. However, those participants that did not lower their BP levels with AEXT may still retain an inherent state of heightened oxidative stress. In the continuum of redox balance, they may still have active compensatory pathways functioning. One such pathway may be the results observed in at-risk African Americans following AEXT. While NO levels increased due to AEXT, VEGF and H_2O_2 levels remained elevated. Thus, elevated VEGF levels functioning as compensation to continue to increase NO production in the face of elevated ROS as demonstrated by increased H_2O_2 levels is one possible explanation for the observed results.

Cellular Experiments: VEGF-mediated eNOS Activity

Detrimental Effects of H_2O_2

To further elucidate the effect of VEGF on NO production, *in vitro* experiments using HUVECs were carried out. Optimal VEGF concentration and length of exposure

were first established. Next, the effects of H_2O_2 on VEGF-mediated signaling were investigated. It was hypothesized that VEGF signaling would be preserved under all *in vitro* experimental conditions; and the activity level of pAkt(473) and peNOS(1177) would be reduced under certain conditions of the proposed model. Indeed, eNOS activity did decrease in a dose dependent fashion to increasing H_2O_2 concentration. A starting H_2O_2 level of 10uM was selected for physiological relevance[175;207;208]. While 10 uM H_2O_2 did decrease VEGF-mediated eNOS activity, it was not until concentrations of 50 and 100 uM of H_2O_2 that the difference in VEGF-mediated eNOS activity became significantly different compared to the no treatment control. A concentration of 10 uM is within the range of circulating human plasma levels. However, higher concentrations are indicative acute inflammatory states such as activated neutrophils[209].

H_2O_2 effects on ECs have demonstrated varied responses. Our results of diminished eNOS activity due to H_2O_2 have also been observed by Sun et al. and Hu et al[210;211]. However, it has also been observed that H_2O_2 increases NO production acutely before subsequent attenuation[212]. Still other researchers have observed an increase in eNOS activity[213]. A definitive explanation of the discrepancy is beyond the scope of the present study.

The model used was an *in vitro* approach to the *in vivo* relationship of oxidative stress on VEGF signaling to the eNOS enzyme. In a translational interpretation, just as hypertensive humans demonstrated elevated oxidative stress, and elevated VEGF levels, they also demonstrate attenuated NO levels, and here we see *in vitro*, that oxidative stress does decrease activity of eNOS under VEGF stimulation.

Akt is a signaling molecule upstream of eNOS activation that is phosphorylated following VEGF stimulation[214]. We did not observe an increase in pAkt activity under the current model. However, it appears that Akt is also activated via serum stimulation[215;216]. The current experimental design did not include a serum starvation period. A possible explanation may be that serum activation may have saturated Akt activity and therefore did not allow for visualization of Akt activity via VEGF stimulation.

Since Akt is a downstream signaling molecule of VEGF, and concurrent increases in pAkt were not observed, another potential mechanism for the observed increase in eNOS activity following VEGF stimulation is AMPK phosphorylation. As discussed previously, the initial increase in eNOS activity induced by VEGF is through calcium influx in the cytosol likely through TRPCs[68] and calmodulin conformation changes[217;218]. VEGF-induced phosphorylation of eNOS at serine 1177 via AMPK is dependent on calcium as well[219].

Atherogenic Flow Conditions are Conducive to VEGF Signaling

After establishing that increasing H_2O_2 levels attenuated VEGF-induced eNOS activity, another detrimental stressor was used to investigate VEGF signaling. An atherogenic LSS level, 4 dyne/cm^2, and an athero-protective LSS level, 20 dyne/cm^2, were applied for 24hrs to African American HUVECs. The creation of these environments using flow-induced phenotypic changes may highlight differences in VEGF-mediated activity following AEXT compared to sedentary states.

We demonstrated that eNOS protein expression increased under athero-protective conditions (20 dyne/cm^2 LSS), which has been well established[220-223]. A significant increase in VEGF-mediated eNOS activity in HUVECs exposed to an atherogenic stimulus was observed. This response has not been characterized previously. In addition, the use of peNOS/eNOS ratio takes into account the increase in eNOS protein level seen after 20 dyne/cm^2. Also, the experimental design considers the known LSS-induced phosphorylation[224] of eNOS by incorporating a non-VEGF stimulated condition for each flow condition.

These results further support the proposed compensatory actions of VEGF under detrimental conditions such as those seen *in vivo*. We observed VEGF-induced eNOS activity was significantly elevated under atherogenic conditions, and *in vivo* it was observed that VEGF levels were elevated in participants with elevated BP at baseline, and also in participants that increased their BP following AEXT.

Under an exercise mimicking and athero-protective LSS level, 20 dyne/cm^2, VEGF-induced eNOS activity was significantly less than atherogenic conditions and *in vivo*, those participants that lowered their BP following AEXT had lower plasma VEGF levels following AEXT. Physiologically, the established increase in eNOS protein that is observed following athero-protective LSS and the decreases in oxidative stress that are known to accompany exercise may also serve to temper the need of VEGF signaling. Perhaps these physiological changes remove the "priming" of the VEGF receptor that is suggested under stressed conditions

To further support these conclusions, we observed a significant decrease in VEGFR2 expression level under athero-protective conditions. It has been established that ECs exhibit beneficial changes in protein expression following 24 hr of athero-protective LSS levels[225-227]. ECs demonstrate a decrease in NADPH oxidase subunits, increases in SOD, GPx, and other antioxidants[228-231]. Perhaps the decrease in VEGFR2 under athero-protective conditions serves to check or regulate the VEGF signaling pathway. It is possible, under athero-protective conditions, VEGF signaling for NO generation is not as critical when there is a redox balance. The necessity of this signaling pathway may not be as prominent when the cellular environment has shifted away from a pro-oxidant state. Though speculative, the regulation of VEGF as a compensatory loop may also be seen in human participants. It was observed that participants with a beneficial lowering of BP level following AEXT also decreased VEGF levels.

Antioxidant expression or activity was not measured in this study. However, it has been observed in previous studies that SOD protein expression[232], and peroxiredoxins[233] increase with athero-protective LSS levels and are elevated in healthy, exercise trained humans[234;235]. Thus, the acute effects of increased ROS may be a trigger for increased eNOS expression as well as other beneficial changes in antioxidant levels. Hence, the shift in the redox balance would be favorable in the long term. Without chronically elevated ROS levels, many compensatory pathways would return to homeostatic levels.

Summary

VEGF activation of its receptor, VEGFR2, activates a myriad of signaling cascades. Of interest however, is the activation of eNOS. We investigated the role that

oxidative stress has on VEGF-mediated eNOS activity and observed that H_2O_2 detrimentally effected eNOS activity in response to VEGF stimulation, which was independent of Akt activity. These *in vitro* observations corresponded to our *in vivo* observations. At baseline, H_2O_2 levels trend upward with increasing BP levels while NO levels trend downward with increasing BP levels. Thus, under conditions of increasing oxidative stress, NO levels are diminished. VEGF levels *in vivo* may be viewed as a compensatory mechanism as they are elevated under higher BP levels and possible elevated oxidative stress. As VEGF does possess redox sensitive transcription factors, this stress response may serve to spur NO production and alleviate oxidative stress[236].

AEXT significantly increased plasma NO levels in the whole population. However, we observed that BP did not decline in the entire population. After separating participants into groups based on their change in BP following AEXT and analyzing their plasma biomarkers, there appeared to be differences. These differences may shed light on the continuum of disease and endothelial status in response to exercise. Participants that lowered their BP also lowered their VEGF levels, and showed no increase in H_2O_2 level. The adaptive response to exercise for its BP-lowering effects is systemic and encompasses many changes from the proteomic level to physiological changes such as vessel remodeling. These beneficial adaptations have likely alleviated the compensatory mechanism of elevated VEGF levels seen at baseline. Indeed, following 24 hr of an athero-protective LSS level, VEGF-mediated eNOS activity was significantly lower compared to 24 hr of LSS at an atherogenic level. The difference in VEGF-mediated

eNOS activity may be due in part to the decrease in VEGFR2 protein expression we observed under an athero-protective LSS level.

The group of participants that increased BP level following AEXT did not experience the same exercise-induced beneficial changes. These differences could have been mediated by differences of genetics, nutrition, or environmental factors. The characteristics of this group demonstrated an increase in VEGF and H_2O_2 levels following AEXT. Hence, the compensatory mechanism was not alleviated in this group, and the proposed inherent feedback loop whereby VEGF levels in stressed systems are increased to compensate for diminished vaso-activity was maintained.

Though participants in this group did complete 6 months of AEXT, changes in BP were not evident. Therefore, the endothelial dysfunction still present in this group may be similar to the experimental atherogenic LSS model. It appears that an atherogenic, 4 dyne/cm^2, LSS level, had significantly higher VEGF-mediated eNOS activity. Under an atherogenic environment, with less eNOS protein, it would be beneficial for activity to be maximized in response to VEGF, a potent stimulator of NO production. Moreover, to aid in VEGF signaling, VEGFR2 protein expression under the atherogenic conditions was significantly higher than athero-protective conditions.

Translational research into characterizing endothelial dysfunction and the progression of CVD is necessary for understanding the complex nature of multi-factorial diseases. Perhaps more important though, is understanding the adaptive processes associated with regression of diseases and chronic oxidative stress. Prevention of disease onset is the new gold standard and comprehending the biological modifications and

regulation associated with both disease progression and regression, along with healthy homeostasis is intrinsic to characterization of complex cellular networks and feedback mechanisms. Perhaps in the future, such work will lend important information for more detailed analysis of personal health.

REFERENCES

1. Kearney PM, Whelton M, Reynolds K et al. Global burden of hypertension: analysis of worldwide data. Lancet 2004;365:217-223.

2. Keehan S, Sisko A, Truffer C et al. Health spending projections through 2017: The baby-boom generation is coming to medicare. Health Affairs 2008;27:W145-W155.

3. American Heart Association. Heart Disease and Stroke Statistics . 2008. Dallas, Texas, American Heart Association.
Ref Type: Generic

4. Gerberding JL. Protecting health - The new research imperative. Jama-Journal of the American Medical Association 2005;294:1403-1406.

5. Cutler JA, Sorlie PD, Wolz M et al. Trends in Hypertension Prevalence, Awareness, Treatment, and Control Rates in United States Adults Between 1988-1994 and 1999-2004. Hypertension 2008;52:818-827.

6. Furchgott RF, Zawadzki JV. The Obligatory Role of Endothelial-Cells in the Relaxation of Arterial Smooth-Muscle by Acetylcholine. Nature 1980;288:373-376.

7. Ignarro LJ, Byrns RE, Buga GM, Wood KS. Endothelium-Derived Relaxing Factor from Pulmonary-Artery and Vein Possesses Pharmacological and Chemical-Properties Identical to Those of Nitric-Oxide Radical. Circ Res 1987;61:866-879.

8. Forstermann U, Closs EI, Pollock JS et al. Nitric-Oxide Synthase Isozymes - Characterization, Purification, Molecular-Cloning, and Functions. Hypertension 1994;23:1121-1131.

9. Radomski MW, Palmer RMJ, Moncada S. An L-Arginine Nitric-Oxide Pathway Present in Human Platelets Regulates Aggregation. Proceedings of the National Academy of Sciences of the United States of America 1990;87:5193-5197.

10. Radomski MW, Palmer RMJ, Moncada S. The Role of Nitric-Oxide and Cgmp in Platelet-Adhesion to Vascular Endothelium. Biochemical and Biophysical Research Communications 1987;148:1482-1489.

11. Kubes P, Suzuki M, Granger DN. Nitric-Oxide - An Endogenous Modulator of Leukocyte Adhesion. Proceedings of the National Academy of Sciences of the United States of America 1991;88:4651-4655.

12. Garg UC, Hassid A. Nitric Oxide-Generating Vasodilators and 8-Bromo-Cyclic Guanosine-Monophosphate Inhibit Mitogenesis and Proliferation of Cultured Rat Vascular Smooth-Muscle Cells. Journal of Clinical Investigation 1989;83:1774-1777.

13. Lockette W, Otsuka Y, Carretero O. The Loss of Endothelium-Dependent Vascular Relaxation in Hypertension. Hypertension 1986;8:61-66.

14. Winquist RJ, Bunting PB, Baskin EP, Wallace AA. Decreased Endothelium-dependent Relaxation in New Zealand Genetic Hypertensive Rats. Journal of Hypertension 1984;2:

15. Ibengwe JK, Suzuki H. Changes in Mechanical Responses of Vascular Smooth Muscles to Acetylcholine, Noradrenaline and High-Potassium Solution in Hypercholesterolemic Rabbits. British Journal of Pharmacology 1986;87:395-402.

16. Verbeuren TJ, Jordaens FH, Zonnekeyn LL et al. Effect of Hypercholesterolemia on Vascular Reactivity in the Rabbit .1. Endothelium-Dependent and Endothelium-Independent Contractions and Relaxations in Isolated Arteries of Control and Hypercholesterolemic Rabbits. Circ Res 1986;58:552-564.

17. Jayakody L, Kappagoda T, Senaratne MPJ, Thomson ABR. Impairment of Endothelium-Dependent Relaxation - An Early Marker for Atherosclerosis in the Rabbit. British Journal of Pharmacology 1988;94:335-346.

18. Egashira K, Suzuki S, Hirooka Y et al. Impaired endothelium-dependent vasodilation of large epicardial and resistance coronary arteries in patients with essential hypertension. Different responses to acetylcholine and substance P. Hypertension 1995;25:201-206.

19. Higashi Y, Oshima T, Ozono R, Matsuura H, Kajiyama G. Aging and severity of hypertension attenuate endothelium-dependent renal vascular relaxation in humans. Hypertension 1997;30:252-258.

20. Levenson JA, Simon AC, Safar ME. Vasodilatation of small and large arteries in hypertension. J.Cardiovasc.Pharmacol. 1985;7 Suppl 2:S115-S120.

21. Papaharalambus CA, Griendling KK. Basic mechanisms of oxidative stress and reactive oxygen species in cardiovascular injury. Trends in Cardiovascular Medicine 2007;17:48-54.

22. Rao GN, Berk BC. Active Oxygen Species Stimulate Vascular Smooth-Muscle Cell-Growth and Protooncogene Expression. Circ Res 1992;70:593-599.

23. Cosentino F, Sill JC, Katusic ZS. Role of Superoxide Anions in the Mediation of Endothelium-Dependent Contractions. Hypertension 1994;23:229-235.

24. Rao GN, Berk BC. Active Oxygen Species Stimulate Vascular Smooth-Muscle Cell-Growth and Protooncogene Expression. Circ Res 1992;70:593-599.

25. Harrison DG. Cellular and molecular mechanisms of endothelial cell dysfunction. Journal of Clinical Investigation 1997;100:2153-2157.

26. Kerr S, Brosnan MJ, McIntyre M et al. Superoxide anion production is increased in a model of genetic hypertension - Role of the endothelium. Hypertension 1999;33:1353-1358.

27. Schnackenberg CG, Wilcox CS. Two-week administration of tempol attenuates both hypertension and renal excretion of 8-iso prostaglandin F-2 alpha. Hypertension 1999;33:424-428.

28. Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases - The role of oxidant stress. Circ Res 2000;87:840-844.

29. Darleyusmar V, Wiseman H, Halliwell B. Nitric-Oxide and Oxygen Radicals - A Question of Balance. FEBS Letters 1995;369:131-135.

30. Stuehr D, Pou S, Rosen GM. Oxygen reduction by nitric-oxide synthases. Journal of Biological Chemistry 2001;276:14533-14536.

31. Pou S, Pou WS, Bredt DS, Snyder SH, Rosen GM. Generation of Superoxide by Purified Brain Nitric-Oxide Synthase. Journal of Biological Chemistry 1992;267:24173-24176.

32. Milstien S, Katusic Z. Oxidation of tetrahydrobiopterin by peroxynitrite: Implications for vascular endothelial function. Biochemical and Biophysical Research Communications 1999;263:681-684.

33. Imaizumi T, Hirooka Y, Masaki H et al. Effects of L-Arginine on Forearm Vessels and Responses to Acetylcholine. Hypertension 1992;20:511-517.

34. Lass A, Suessenbacher A, Wolkart G, Mayer B, Brunner F. Functional and analytical evidence for scavenging of oxygen radicals by L-arginine. Molecular Pharmacology 2002;61:1081-1088.

35. Wassmann S, Wassmann K, Nickenig G. Modulation of oxidant and antioxidant enzyme expression and function in vascular cells. Hypertension 2004;44:381-386.

36. Michiels C, Raes M, Toussaint O, Remacle J. Importance of Se-Glutathione Peroxidase, Catalase, and Cu/Zn-Sod for Cell-Survival Against Oxidative Stress. Free Radical Biology and Medicine 1994;17:235-248.

37. Abedi H, Zachary I. Vascular endothelial growth factor stimulates tyrosine phosphorylation and recruitment to new focal adhesions of focal adhesion kinase and paxillin in endothelial cells. Journal of Biological Chemistry 1997;272:15442-15451.

38. Harlan JM, Levine JD, Callahan KS, Schwartz BR, Harker LA. Glutathione Redox Cycle Protects Cultured Endothelial-Cells Against Lysis by Extracellularly Generated Hydrogen-Peroxide. Journal of Clinical Investigation 1984;73:706-713.

39. Roos D, Weening RS, Voetman AA et al. Protection of Phagocytic Leukocytes by Endogenous Glutathione - Studies in A Family with Glutathione-Reductase Deficiency. Blood 1979;53:851-866.

40. Wei MH, Popescu NC, Lerman MI, Merrill MJ, Zimonjic DB. Localization of the human vascular endothelial growth factor gene, VEGF at chromosome 6p12. Human Genetics 1996;97:794-797.

41. Poltorak Z, Cohen T, Sivan R et al. VEGF(145), a secreted vascular endothelial growth factor isoform that binds to extracellular matrix. Journal of Biological Chemistry 1997;272:7151-7158.

42. Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z. Vascular endothelial growth factor (VEGF) and its receptors. Faseb Journal 1999;13:9-22.

43. Tischer E, Mitchell R, Hartman T et al. The Human Gene for Vascular Endothelial Growth-Factor - Multiple Protein Forms Are Encoded Through Alternative Exon Splicing. Journal of Biological Chemistry 1991;266:11947-11954.

44. Fairbrother WJ, Champe MA, Christinger HW, Keyt BA, Starovasnik MA. Solution structure of the heparin-binding domain of vascular endothelial growth factor. Structure 1998;6:637-648.

45. Houck KA, Leung DW, Rowland AM, Winer J, Ferrara N. Dual Regulation of Vascular Endothelial Growth-Factor Bioavailability by Genetic and Proteolytic Mechanisms. Journal of Biological Chemistry 1992;267:26031-26037.

46. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular Endothelial Growth-Factor Is A Secreted Angiogenic Mitogen. Science 1989;246:1306-1309.

47. Ferrara N. Molecular and biological properties of vascular endothelial growth factor. Journal of Molecular Medicine-Jmm 1999;77:527-543.

48. Bunn HF, Poyton RO. Oxygen sensing and molecular adaptation to hypoxia. Physiological Reviews 1996;76:839-885.

49. Shweiki D, Itin A, Soffer D, Keshet E. Vascular Endothelial Growth-Factor Induced by Hypoxia May Mediate Hypoxia-Initiated Angiogenesis. Nature 1992;359:843-845.

50. Ladoux A, Frelin C. Hypoxia Is A Strong Inducer of Vascular Endothelial Growth-Factor Messenger-Rna Expression in the Heart. Biochemical and Biophysical Research Communications 1993;195:1005-1010.

51. Levy AP, Levy NS, Wegner S, Goldberg MA. Transcriptional Regulation of the Rat Vascular Endothelial Growth-Factor Gene by Hypoxia. Journal of Biological Chemistry 1995;270:13333-13340.

52. Jaakkola P, Mole DR, Tian YM et al. Targeting of HIF-alpha to the von Hippel-Lindau Ubiquitylation Complex by O2-Regulated Prolyl Hydroxylation. Science 2001;292:468-472.

53. Millauer B, Wizigmannvoos S, Schnurch H et al. High-Affinity Vegf Binding and Developmental Expression Suggest Flk-1 As A Major Regulator of Vasculogenesis and Angiogenesis. Cell 1993;72:835-846.

54. Soker S, Takashima S, Miao HQ, Neufeld G, Klagsbrun M. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. Cell 1998;92:735-745.

55. Otrock ZK, Makarem JA, Shamseddine AI. Vascular endothelial growth factor family of ligands and receptors: review. Blood Cells Mol.Dis. 2007;38:258-268.

56. Takahashi T, Shibuya M. The 230 kDa mature form of KDR/Flk-1 (VEGF receptor-2) activates the PLC-gamma pathway and partially induces mitotic signals in NIH3T3 fibroblasts. Oncogene 1997;14:2079-2089.

57. Jin ZG, Ueba H, Tanimoto T et al. Ligand-Independent Activation of Vascular Endothelial Growth Factor Receptor 2 by Fluid Shear Stress Regulates Activation of Endothelial Nitric Oxide Synthase. Circ Res 2003;93:354-363.

58. Zachary I, Gliki G. Signaling transduction mechanisms mediating biological actions of the vascular endothelial growth factor family. Cardiovascular Research 2001;49:568-581.

59. Gerber HP, McMurtrey A, Kowalski J et al. Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3 '-kinase Akt signal transduction pathway - Requirement for Flk-1/KDR activation. Journal of Biological Chemistry 1998;273:30336-30343.

60. Gerber HP, Dixit V, Ferrara N. Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. Journal of Biological Chemistry 1998;273:13313-13316.

61. Tran J, Rak J, Sheehan C et al. Marked induction of the IAP family antiapoptotic proteins survivin and XIAP by VEGF in vascular endothelial cells. Biochemical and Biophysical Research Communications 1999;264:781-788.

62. Abedi H, Zachary I. Vascular endothelial growth factor stimulates tyrosine phosphorylation and recruitment to new focal adhesions of focal adhesion kinase and paxillin in endothelial cells. Journal of Biological Chemistry 1997;272:15442-15451.

63. Wheeler-Jones C, bu-Ghazaleh R, Cospedal R et al. Vascular endothelial growth factor stimulates prostacyclin production and activation of cytosolic phospholipase A2 in endothelial cells via p42/p44 mitogen-activated protein kinase. FEBS Letters 1997;420:28-32.

64. Abedi H, Zachary I. Vascular endothelial growth factor stimulates tyrosine phosphorylation and recruitment to new focal adhesions of focal adhesion kinase and paxillin in endothelial cells. Journal of Biological Chemistry 1997;272:15442-15451.

65. Ku DD, Zaleski JK, Liu SY, Brock TA. Vascular Endothelial Growth-Factor Induces Edrf-Dependent Relaxation in Coronary-Arteries. American Journal of Physiology 1993;265:H586-H592.

66. Horowitz JR, Rivard A, vanderZee R et al. Vascular endothelial growth factor vascular permeability factor produces nitric oxide-dependent hypotension - Evidence for a maintenance role in quiescent adult endothelium. Arteriosclerosis Thrombosis and Vascular Biology 1997;17:2793-2800.

67. Hood JD, Meininger CJ, Ziche M, Granger HJ. VEGF upregulates ecNOS message, protein, and NO production in human endothelial cells. American Journal of Physiology-Heart and Circulatory Physiology 1998;43:H1054-H1058.

68. Hamdollah Zadeh MA, Glass C, Magnussen A, Hancox J, Bates D. VEGF-Mediated Elevated Intracellular Calcium and Angiogenesis in Human Microvascular Endothelial Cells In Vitro are Inhibited by Dominant Negative TRPC6. Microcirculation 2008;15:605-614.

69. Chin D, Means AR. Calmodulin: a prototypical calcium sensor. Trends Cell Biol 2000;10:322-328.

70. Greif DM, Sacks DB, Michel T. Calmodulin phosphorylation and modulation of endothelial nitric oxide synthase catalysis. Proc.Natl.Acad.Sci.U.S A 2004;101:1165-1170.

71. Feliers D, Chen X, Akis N et al. VEGF regulation of endothelial nitric oxide synthase in glomerular endothelial cells. Kidney Int. 2005;68:1648-1659.

72. Reihill JA, Ewart MA, Hardie DG, Salt IP. AMP-activated protein kinase mediates VEGF-stimulated endothelial NO production. Biochem.Biophys.Res Commun. 2007;354:1084-1088.

73. Eppler SM, Combs DL, Henry TD et al. A target-mediated model to describe the pharmacokinetics and hemodynamic effects of recombinant human vascular endothelial growth factor in humans. Clinical Pharmacology & Therapeutics 2002;72:20-32.

74. Gelinas DS, Bernatchez PN, Rollin S, Bazan NG, Sirois MG. Immediate and delayed VEGF-mediated NO synthesis in endothelial cells: Role of PI3K, PKC and PLC pathways. British Journal of Pharmacology 2002;137:1021-1030.

75. Wheeler-Jones C, bu-Ghazaleh R, Cospedal R et al. Vascular endothelial growth factor stimulates prostacyclin production and activation of cytosolic phospholipase A2 in endothelial cells via p42/p44 mitogen-activated protein kinase. FEBS Letters 1997;420:28-32.

76. Chowdhury S, Spicer JF, Harper PG. Hypertension and targeted therapy Part 2: Small molecule inhibitors of VEGF. Targeted Oncology 2006;1:172-178.

77. Chowdhury S, Spicer JF, Harper PG. Hypertension and targeted therapy Part 1: Bevacizumab. Targeted Oncology 2006;1:104-108.

78. Jain RK, Duda DG, Clark JW, Loeffler JS. Lessons from phase III clinical trials on anti-VEGF therapy for cancer. Nature Clinical Practice Oncology 2006;3:24-40.

79. Curwen JO, Musgrove HL, Kendrew J et al. Inhibition of vascular endothelial growth factor-a signaling induces hypertension: examining the effect of

cediranib (recentin; AZD2171) treatment on blood pressure in rat and the use of concomitant antihypertensive therapy. Clin.Cancer Res 2008;14:3124-3131.

80. Stumpf C, Jukic J, Yilmaz A et al. Elevated VEGF-plasma levels in young patients with mild essential hypertension. Eur.J.Clin.Invest 2009;39:31-36.

81. Nadar SK, Blann AD, Lip GY. Plasma and platelet-derived vascular endothelial growth factor and angiopoietin-1 in hypertension: effects of antihypertensive therapy. J.Intern.Med. 2004;256:331-337.

82. Nadar SK, Blann A, Beevers DG, Lip GYH. Abnormal angiopoietins 1&2, angiopoietin receptor Tie-2 and vascular endothelial growth factor levels in hypertension: relationship to target organ damage [a sub-study of the Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT)]. Journal of Internal Medicine 2005;258:336-343.

83. Blann AD, Belgore FM, Constans J, Conri C, Lip GYP. Plasma vascular endothelial growth factor and its receptor Flt-1 in patients with hyperlipidemia and atherosclerosis and the effects of Fluvastatin or Fenofibrate. American Journal of Cardiology 2001;87:1160-1163.

84. Chin BSP, Chung NAY, Gibbs CR, Blann AD, Lip GYH. Vascular endothelial growth factor and soluble P-selectin in acute and chronic congestive heart failure. American Journal of Cardiology 2002;90:1258-+.

85. Miyazawa-Hoshimoto S, Takahashi K, Bujo H, Hashimoto N, Saito Y. Elevated serum vascular endothelial growth factor is associated with visceral fat accumulation in human obese subjects. Diabetologia 2003;46:1483-1488.

86. Blann AD, Belgore FM, McCollum CN et al. Vascular endothelial growth factor and its receptor, Flt-l, in the plasma of patients with coronary or peripheral atherosclerosis, or type II diabetes. Clinical Science 2002;102:187-194.

87. Viazzi F, Leoncini G, Ratto E et al. Vascular permeability, blood pressure, and organ damage in primary hypertension. Journal of Hypertension 2007;25:S107.

88. Eremina V, Sood M, Haigh J et al. Glomerular-specific alterations of VEGF-A expression lead to distinct congenital and acquired renal diseases. Journal of Clinical Investigation 2003;111:707-716.

89. Booth FW, Chakravarthy MV, Spangenburg EE. Exercise and gene expression: physiological regulation of the human genome through physical activity. Journal of Physiology-London 2002;543:399-411.

90. Chobanian AV, Bakris GL, Black HR et al. The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure: the JNC 7 report. JAMA 2003;289:2560-2572.

91. U.S.Department of Health and Human Services. National Health and Nutrition Examination Survey Data. Centers for Disease Control and Prevention. 1999. Hyattsville, MD. 2004.
Ref Type: Generic

92. Goto C, Higashi Y, Kimura M et al. Effect of different intensities of exercise on endothelium-dependent vasodilation in humans - Role of endothelium-dependent nitric oxide and oxidative stress. Circulation 2003;108:530-535.

93. Malek AM, Alper SL, Izumo S. Hemodynamic shear stress and its role in atherosclerosis. JAMA 1999;282:2035-2042.

94. Husain K. Interaction of physical training and chronic nitroglycerin treatment on blood pressure, nitric oxide, and oxidants/antioxidants in the rat heart. Pharmacological Research 2003;48:253-261.

95. Park JY, Ferrell RE, Park JJ et al. NADPH oxidase p22phox gene variants are associated with systemic oxidative stress biomarker responses to exercise training. Journal of Applied Physiology 2005;99:1905-1911.

96. Inoue N, Ramasamy S, Fukai T, Nerem RM, Harrison DG. Shear stress modulates expression of Cu/Zn superoxide dismutase in human aortic endothelial gels. Circ Res 1996;79:32-37.

97. Green DJ, Maiorana A, O'Driscoll G, Taylor R. Effect of exercise training on endothelium-derived nitric oxide function in humans. Journal of Physiology-London 2004;561:1-25.

98. Kohno S, Kawai Y, Wada C, Gemba M. NO generation and mitochondrial membrane potential loss in hypoxic injury of isolated rat renal tubules. Japanese Journal of Pharmacology 2002;88:205P.

99. Goto C, Higashi Y, Kimura M et al. Effect of different intensities of exercise on endothelium-dependent vasodilation in humans - Role of endothelium-dependent nitric oxide and oxidative stress. Circulation 2003;108:530-535.

100. Miller VM, Vanhoutte PM. Enhanced release of endothelium-derived factor(s) by chronic increases in blood flow. Am.J.Physiol 1988;255:H446-H451.

101. Rush JWE, Denniss SG, Graham DA. Vascular nitric oxide and oxidative stress: Determinants of endothelial adaptations to cardiovascular disease and to

physical activity. Canadian Journal of Applied Physiology-Revue Canadienne de Physiologie Appliquee 2005;30:442-474.

102. Maeda S, Tanabe T, Tsuk T et al. Moderate regular exercise increases basal production of nitric oxide in elderly women. Hypertension Research 2004;27:947-953.

103. Inoue N, Ramasamy S, Fukai T, Nerem RM, Harrison DG. Shear stress modulates expression of Cu/Zn superoxide dismutase in human aortic endothelial gels. Circ Res 1996;79:32-37.

104. Takeshita S, Inoue N, Ueyama T, Kawashima S, Yokoyama M. Shear stress enhances glutathione peroxidase expression in endothelial cells. Biochemical and Biophysical Research Communications 2000;273:66-71.

105. Wilson DO, Johnson P. Exercise modulates antioxidant enzyme gene expression in rat myocardium and liver. Journal of Applied Physiology 2000;88:1791-1796.

106. Malek AM, Alper SL, Izumo S. Hemodynamic shear stress and its role in atherosclerosis. JAMA 1999;282:2035-2042.

107. Zarins CK, Giddens DP, Bharadvaj BK et al. Carotid bifurcation atherosclerosis. Quantitative correlation of plaque localization with flow velocity profiles and wall shear stress. Circ Res 1983;53:502-514.

108. Traub O, Berk BC. Laminar shear stress: mechanisms by which endothelial cells transduce an atheroprotective force. Arterioscler.Thromb.Vasc.Biol. 1998;18:677-685.

109. Davis ME, Cai H, McCann L, Fukai T, Harrison DG. Role of c-Src in regulation of endothelial nitric oxide synthase expression during exercise training. American Journal of Physiology-Heart and Circulatory Physiology 2003;284:H1449-H1453.

110. Lan QX, Mercurius KO, Davies PF. Stimulation of Transcription Factors NF[kappa]B and AP1 in Endothelial Cells Subjected to Shear Stress. Biochemical and Biophysical Research Communications 1994;201:950-956.

111. Traub O, Berk BC. Laminar shear stress: mechanisms by which endothelial cells transduce an atheroprotective force. Arterioscler.Thromb.Vasc.Biol. 1998;18:677-685.

112. Chen XL, Varner SE, Rao AS et al. Laminar Flow Induction of Antioxidant Response Element-mediated Genes in Endothelial Cells. Journal of Biological Chemistry 2003;278:703-711.

113. Sessa WC, Pritchard K, Seyedi N, Wang J, Hintze TH. Chronic exercise in dogs increases coronary vascular nitric oxide production and endothelial cell nitric oxide synthase gene expression. Circ Res 1994;74:349-353.

114. Higashi Y, Yoshizumi M. Exercise and endothelial function: role of endothelium-derived nitric oxide and oxidative stress in healthy subjects and hypertensive patients. Pharmacol.Ther. 2004;102:87-96.

115. Bray MS. Genomics, genes, and environmental interaction: the role of exercise. J.Appl.Physiol 2000;88:788-792.

116. Park JY, Farrance IK, Fenty NM et al. NFKB1 promoter variation implicates shear-induced NOS3 gene expression and endothelial function in prehypertensives and stage I hypertensives. Am.J.Physiol Heart Circ.Physiol 2007;293:H2320-H2327.

117. Malek AM, Alper SL, Izumo S. Hemodynamic shear stress and its role in atherosclerosis. JAMA 1999;282:2035-2042.

118. Duerrschmidt N, Stielow C, Muller G, Pagano PJ, Morawietz H. NO-mediated regulation of NAD(P)H oxidase by laminar shear stress in human endothelial cells. J.Physiol 2006;576:557-567.

119. Wasserman SM, Mehraban F, Komuves LG et al. Gene expression profile of human endothelial cells exposed to sustained fluid shear stress. Physiol Genomics 2002;12:13-23.

120. Topper JN, Cai J, Falb D, Gimbrone MA, Jr. Identification of vascular endothelial genes differentially responsive to fluid mechanical stimuli: cyclooxygenase-2, manganese superoxide dismutase, and endothelial cell nitric oxide synthase are selectively up-regulated by steady laminar shear stress. Proc.Natl.Acad.Sci.U.S A 1996;93:10417-10422.

121. Inoue N, Ramasamy S, Fukai T, Nerem RM, Harrison DG. Shear stress modulates expression of Cu/Zn superoxide dismutase in human aortic endothelial gels. Circ Res 1996;79:32-37.

122. Chen XL, Varner SE, Rao AS et al. Laminar flow induction of antioxidant response element-mediated genes in endothelial cells. A novel anti-inflammatory mechanism. J Biol Chem. 2003;278:703-711.

123. Mowbray AL, Kang DH, Rhee SG, Kang SW, Jo H. Laminar shear stress up-regulates peroxiredoxins (PRX) in endothelial cells: PRX 1 as a mechanosensitive antioxidant. J Biol Chem. 2008;283:1622-1627.

124. Conklin BS, Zhong DS, Zhao WD, Lin PH, Chen CY. Shear stress regulates occludin and VEGF expression in porcine arterial endothelial cells. Journal of Surgical Research 2002;102:13-21.

125. Chen KD, Li YS, Kim M et al. Mechanotransduction in response to shear stress. Roles of receptor tyrosine kinases, integrins, and Shc. J Biol Chem. 1999;274:18393-18400.

126. Jin ZG, Ueba H, Tanimoto T et al. Ligand-independent activation of vascular endothelial growth factor receptor 2 by fluid shear stress regulates activation of endothelial nitric oxide synthase. Circ Res 2003;93:354-363.

127. Jin ZG, Ueba H, Tanimoto T et al. Ligand-independent activation of vascular endothelial growth factor receptor 2 by fluid shear stress regulates activation of endothelial nitric oxide synthase. Circ Res 2003;93:354-363.

128. Urbich C, Stein M, Reisinger K et al. Fluid shear stress-induced transcriptional activation of the vascular endothelial growth factor receptor-2 gene requires Sp1-dependent DNA binding. FEBS Letters 2003;535:87-93.

129. Khatri JJ, Johnson C, Magid R et al. Vascular oxidant stress enhances progression and angiogenesis of experimental atheroma. Circulation 2004;109:520-525.

130. Yang H, Roberts LJ, Shi MJ et al. Retardation of atherosclerosis by overexpression of catalase or both Cu/Zn-superoxide dismutase and catalase in mice lacking apolipoprotein E. Circ Res 2004;95:1075-1081.

131. Tribble DL, Gong EL, Leeuwenburgh C et al. Fatty streak formation in fat-fed mice expressing human copper-zinc superoxide dismutase. Arterioscler Thromb Vasc Biol 1997;17:1734-1740.

132. Karolkiewicz J, Szczesniak L, skur-Smielecka E et al. Oxidative stress and antioxidant defense system in healthy, elderly men: relationship to physical activity. Aging Male. 2003;6:100-105.

133. Lacy F, O'Connor DT, Schmid-Schonbein GW. Plasma hydrogen peroxide production in hypertensives and normotensive subjects at genetic risk of hypertension. Journal of Hypertension 1998;16:291-303.

134. Deskur E, Przywarska I, Dylewicz P et al. Exercise-induced increase in hydrogen peroxide plasma levels is diminished by endurance training after myocardial infarction. Int.J.Cardiol. 1998;67:219-224.

135. Horoz M, Bolukbas C, Bolukbas FF et al. Oxidative stress in hepatitis C infected end-stage renal disease subjects. BMC Infect.Dis. 2006;6:114.

136. Gonzalez-Pacheco FR, Deudero JJP, Castellanos MC et al. Mechanisms of endothelial response to oxidative aggression: Protective role of autologous VEGF and induction of VEGFR2 by H2O2. American Journal of Physiology-Heart and Circulatory Physiology 2006;291:H1395-H1401.

137. Cai H, Li Z, Dikalov S et al. NAD(P)H oxidase-derived hydrogen peroxide mediates endothelial nitric oxide production in response to angiotensin II. J Biol Chem. 2002;277:48311-48317.

138. Fujita H, Morita I, Murota S. Hydrogen peroxide induced apoptosis of endothelial cells concomitantly with cycloheximide. J Atheroscler.Thromb 2000;7:209-215.

139. Tian J, Hou Y, Lu Q et al. A novel role for caveolin-1 in regulating endothelial nitric oxide synthase activation in response to H2O2 and shear stress. Free Radic.Biol Med 2010;49:159-170.

140. Boulden BM, Widder JD, Allen JC et al. Early determinants of H2O2-induced endothelial dysfunction. Free Radic.Biol Med 2006;41:810-817.

141. Droge W. Free radicals in the physiological control of cell function. Physiol Rev. 2002;82:47-95.

142. Antunes F, Cadenas E. Estimation of H2O2 gradients across biomembranes. FEBS Lett. 2000;475:121-126.

143. Colavitti R, Pani G, Bedogni B et al. Reactive oxygen species as downstream mediators of angiogenic signaling by vascular endothelial growth factor receptor-2/KDR. J Biol Chem. 2002;277:3101-3108.

144. Bokoch GM, Diebold BA. Current molecular models for NADPH oxidase regulation by Rac GTPase. Blood 2002;100:2692-2696.

145. Radi R. Nitric oxide, oxidants, and protein tyrosine nitration. Proc.Natl.Acad.Sci.U.S A 2004;101:4003-4008.

146. Mondoro TH, Shafer BC, Vostal JG. Peroxynitrite-induced tyrosine nitration and phosphorylation in human platelets. Free Radic.Biol Med 1997;22:1055-1063.

147. Mallozzi C, De FL, Brugnara C, Di Stasi AM. Protein phosphatase 1alpha is tyrosine-phosphorylated and inactivated by peroxynitrite in erythrocytes through the src family kinase fgr. Free Radic.Biol Med 2005;38:1625-1636.

148. Mallozzi C, Di Stasi AM, Minetti M. Nitrotyrosine mimics phosphotyrosine binding to the SH2 domain of the src family tyrosine kinase lyn. FEBS Lett. 2001;503:189-195.

149. Mallozzi C, Di Stasi MA, Minetti M. Peroxynitrite-dependent activation of src tyrosine kinases lyn and hck in erythrocytes is under mechanistically different pathways of redox control. Free Radic.Biol Med 2001;30:1108-1117.

150. Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. Physiol Rev. 2007;87:315-424.

151. Szabo C, Ischiropoulos H, Radi R. Peroxynitrite: biochemistry, pathophysiology and development of therapeutics. Nat.Rev.Drug Discov. 2007;6:662-680.

152. Oshikawa J, Urao N, Kim HW et al. Extracellular SOD-derived H2O2 promotes VEGF signaling in caveolae/lipid rafts and post-ischemic angiogenesis in mice. PLoS One. 2010;5:e10189.

153. Drummond GR, Cai H, Davis ME, Ramasamy S, Harrison DG. Transcriptional and posttranscriptional regulation of endothelial nitric oxide synthase expression by hydrogen peroxide. Circ Res 2000;86:347-354.

154. Kumar S, Sun X, Wiseman DA et al. Hydrogen peroxide decreases endothelial nitric oxide synthase promoter activity through the inhibition of Sp1 activity. DNA Cell Biol 2009;28:119-129.

155. Cai H, Davis ME, Drummond GR, Harrison DG. Induction of endothelial NO synthase by hydrogen peroxide via a Ca(2+)/calmodulin-dependent protein kinase II/janus kinase 2-dependent pathway. Arterioscler Thromb Vasc Biol 2001;21:1571-1576.

156. Lauer N, Suvorava T, Ruther U et al. Critical involvement of hydrogen peroxide in exercise-induced up-regulation of endothelial NO synthase. Cardiovasc.Res 2005;65:254-262.

157. Hu Z, Chen J, Wei Q, Xia Y. Bidirectional actions of hydrogen peroxide on endothelial nitric-oxide synthase phosphorylation and function: co-commitment and interplay of Akt and AMPK. J Biol Chem. 2008;283:25256-25263.

158. Boulden BM, Widder JD, Allen JC et al. Early determinants of H2O2-induced endothelial dysfunction. Free Radic.Biol Med 2006;41:810-817.

159. Tampo Y, Kotamraju S, Chitambar CR et al. Oxidative stress-induced iron signaling is responsible for peroxide-dependent oxidation of dichlorodihydrofluorescein in endothelial cells: role of transferrin receptor-dependent iron uptake in apoptosis. Circ Res 2003;92:56-63.

160. Thomas SR, Schulz E, Keaney JF, Jr. Hydrogen peroxide restrains endothelium-derived nitric oxide bioactivity -- role for iron-dependent oxidative stress. Free Radic.Biol Med 2006;41:681-688.

161. Thomas SR, Witting PK, Drummond GR. Redox control of endothelial function and dysfunction: molecular mechanisms and therapeutic opportunities. Antioxid.Redox Signal 2008;10:1713-1765.

162. Coyle CH, Martinez LJ, Coleman MC et al. Mechanisms of H2O2-induced oxidative stress in endothelial cells. Free Radic.Biol Med 2006;40:2206-2213.

163. Djordjevic T, Pogrebniak A, BelAiba RS et al. The expression of the NADPH oxidase subunit p22phox is regulated by a redox-sensitive pathway in endothelial cells. Free Radic.Biol Med 2005;38:616-630.

164. Witting PK, Rayner BS, Wu BJ, Ellis NA, Stocker R. Hydrogen peroxide promotes endothelial dysfunction by stimulating multiple sources of superoxide anion radical production and decreasing nitric oxide bioavailability. Cell Physiol Biochem. 2007;20:255-268.

165. Coyle CH, Martinez LJ, Coleman MC et al. Mechanisms of H2O2-induced oxidative stress in endothelial cells. Free Radic.Biol Med 2006;40:2206-2213.

166. Witting PK, Rayner BS, Wu BJ, Ellis NA, Stocker R. Hydrogen peroxide promotes endothelial dysfunction by stimulating multiple sources of superoxide anion radical production and decreasing nitric oxide bioavailability. Cell Physiol Biochem. 2007;20:255-268.

167. Chen K, Thomas SR, Albano A, Murphy MP, Keaney JF, Jr. Mitochondrial function is required for hydrogen peroxide-induced growth factor receptor transactivation and downstream signaling. J Biol Chem. 2004;279:35079-35086.

168. Hood JD, Meininger CJ, Ziche M, Granger HJ. VEGF upregulates ecNOS message, protein, and NO production in human endothelial cells.

American Journal of Physiology-Heart and Circulatory Physiology 1998;43:H1054-H1058.

169. Horowitz JR, Rivard A, vanderZee R et al. Vascular endothelial growth factor vascular permeability factor produces nitric oxide-dependent hypotension - Evidence for a maintenance role in quiescent adult endothelium. Arteriosclerosis Thrombosis and Vascular Biology 1997;17:2793-2800.

170. Ku DD, Zaleski JK, Liu SY, Brock TA. Vascular Endothelial Growth-Factor Induces Edrf-Dependent Relaxation in Coronary-Arteries. American Journal of Physiology 1993;265:H586-H592.

171. Chowdhury S, Spicer JF, Harper PG. Hypertension and targeted therapy Part 2: Small molecule inhibitors of VEGF. Targeted Oncology 2006;1:172-178.

172. Chowdhury S, Spicer JF, Harper PG. Hypertension and targeted therapy Part 1: Bevacizumab. Targeted Oncology 2006;1:104-108.

173. Jain RK, Duda DG, Clark JW, Loeffler JS. Lessons from phase III clinical trials on anti-VEGF therapy for cancer. Nature Clinical Practice Oncology 2006;3:24-40.

174. Deskur E, Przywarska I, Dylewicz P et al. Exercise-induced increase in hydrogen peroxide plasma levels is diminished by endurance training after myocardial infarction. Int.J.Cardiol. 1998;67:219-224.

175. Horoz M, Bolukbas C, Bolukbas FF et al. Oxidative stress in hepatitis C infected end-stage renal disease subjects. BMC Infect.Dis. 2006;6:114.

176. Karolkiewicz J, Szczesniak L, skur-Smielecka E et al. Oxidative stress and antioxidant defense system in healthy, elderly men: relationship to physical activity. Aging Male. 2003;6:100-105.

177. Lacy F, O'Connor DT, Schmid-Schonbein GW. Plasma hydrogen peroxide production in hypertensives and normotensive subjects at genetic risk of hypertension. Journal of Hypertension 1998;16:291-303.

178. Gonzalez-Pacheco FR, Deudero JJP, Castellanos MC et al. Mechanisms of endothelial response to oxidative aggression: Protective role of autologous VEGF and induction of VEGFR2 by H2O2. American Journal of Physiology-Heart and Circulatory Physiology 2006;291:H1395-H1401.

179. Nadar SK, Blann A, Beevers DG, Lip GYH. Abnormal angiopoietins 1&2, angiopoietin receptor Tie-2 and vascular endothelial growth factor levels in hypertension: relationship to target organ damage [a sub-study of the

Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT)]. Journal of Internal Medicine 2005;258:336-343.

180. Stumpf C, Jukic J, Yilmaz A et al. Elevated VEGF-plasma levels in young patients with mild essential hypertension. Eur.J.Clin.Invest 2009;39:31-36.

181. Chin BSP, Chung NAY, Gibbs CR, Blann AD, Lip GYH. Vascular endothelial growth factor and soluble P-selectin in acute and chronic congestive heart failure. American Journal of Cardiology 2002;90:1258-+.

182. Nadar SK, Blann A, Beevers DG, Lip GYH. Abnormal angiopoietins 1&2, angiopoietin receptor Tie-2 and vascular endothelial growth factor levels in hypertension: relationship to target organ damage [a sub-study of the Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT)]. Journal of Internal Medicine 2005;258:336-343.

183. Stumpf C, Jukic J, Yilmaz A et al. Elevated VEGF-plasma levels in young patients with mild essential hypertension. Eur.J.Clin.Invest 2009;39:31-36.

184. Gerber HP, McMurtrey A, Kowalski J et al. Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3 '-kinase Akt signal transduction pathway - Requirement for Flk-1/KDR activation. Journal of Biological Chemistry 1998;273:30336-30343.

185. Oshikawa J, Urao N, Kim HW et al. Extracellular SOD-derived H2O2 promotes VEGF signaling in caveolae/lipid rafts and post-ischemic angiogenesis in mice. PLoS One. 2010;5:e10189.

186. Perkin Elmer. Perkin Elmer Surefire Technology, Endogenous Kinase Activity. 2009.
Ref Type: Serial (Book,Monograph)

187. Dossumbekova A, Berdyshev EV, Gorshkova I et al. Akt activates NOS3 and separately restores barrier integrity in H2O2-stressed human cardiac microvascular endothelium. Am.J Physiol Heart Circ Physiol 2008;295:H2417-H2426.

188. Gratton JP, Morales-Ruiz M, Kureishi Y et al. Akt down-regulation of p38 signaling provides a novel mechanism of vascular endothelial growth factor-mediated cytoprotection in endothelial cells. J Biol Chem. 2001;276:30359-30365.

189. Thomas SR, Chen K, Keaney JF, Jr. Hydrogen peroxide activates endothelial nitric-oxide synthase through coordinated phosphorylation and dephosphorylation via a phosphoinositide 3-kinase-dependent signaling pathway. J Biol Chem. 2002;277:6017-6024.

190. Ferrara N. Molecular and biological properties of vascular endothelial growth factor. Journal of Molecular Medicine-Jmm 1999;77:527-543.

191. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular Endothelial Growth-Factor Is A Secreted Angiogenic Mitogen. Science 1989;246:1306-1309.

192. Abedi H, Zachary I. Vascular endothelial growth factor stimulates tyrosine phosphorylation and recruitment to new focal adhesions of focal adhesion kinase and paxillin in endothelial cells. Journal of Biological Chemistry 1997;272:15442-15451.

193. Eppler SM, Combs DL, Henry TD et al. A target-mediated model to describe the pharmacokinetics and hemodynamic effects of recombinant human vascular endothelial growth factor in humans. Clinical Pharmacology & Therapeutics 2002;72:20-32.

194. Ku DD, Zaleski JK, Liu SY, Brock TA. Vascular Endothelial Growth-Factor Induces Edrf-Dependent Relaxation in Coronary-Arteries. American Journal of Physiology 1993;265:H586-H592.

195. Liu MH, Jin HK, Floten HS et al. Vascular endothelial growth factor-mediated endothelium-dependent relaxation is blunted in spontaneously hypertensive rats. J.Pharmacol.Exp.Ther. 2001;296:473-477.

196. Lockette W, Otsuka Y, Carretero O. The Loss of Endothelium-Dependent Vascular Relaxation in Hypertension. Hypertension 1986;8:61-66.

197. Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. Physiol Rev. 2007;87:315-424.

198. Roy S, Khanna S, Sen CK. Redox regulation of the VEGF signaling path and tissue vascularization: Hydrogen peroxide, the common link between physical exercise and cutaneous wound healing. Free Radic.Biol Med 2008;44:180-192.

199. Tsurumi Y, Murohara T, Krasinski K et al. Reciprocal relation between VEGF and NO in the regulation of endothelial integrity. Nature Medicine 1997;3:879-886.

200. Kroll J, Waltenberger J. VEGF-A induces expression of eNOS and iNOS in endothelial cells via VEGF receptor-2 (KDR). Biochem.Biophys.Res Commun. 1998;252:743-746.

201. Reihill JA, Ewart MA, Hardie DG, Salt IP. AMP-activated protein kinase mediates VEGF-stimulated endothelial NO production. Biochem.Biophys.Res Commun. 2007;354:1084-1088.

202. Shen BQ, Lee DY, Zioncheck TF. Vascular endothelial growth factor governs endothelial nitric-oxide synthase expression via a KDR/Flk-1 receptor and a protein kinase C signaling pathway. J Biol Chem. 1999;274:33057-33063.

203. Tsurumi Y, Murohara T, Krasinski K et al. Reciprocal relation between VEGF and NO in the regulation of endothelial integrity. Nature Medicine 1997;3:879-886.

204. Booth FW, Vyas DR. Genes, environment, and exercise. Hypoxia: from Genes to the Bedside 2001;502:13-20.

205. Green DJ, Maiorana A, O'Driscoll G, Taylor R. Effect of exercise training on endothelium-derived nitric oxide function in humans. Journal of Physiology-London 2004;561:1-25.

206. Paffenbarger RS, Jr., Hyde RT, Hsieh CC, Wing AL. Physical activity, other life-style patterns, cardiovascular disease and longevity. Acta Med.Scand.Suppl 1986;711:85-91.

207. Deskur E, Przywarska I, Dylewicz P et al. Exercise-induced increase in hydrogen peroxide plasma levels is diminished by endurance training after myocardial infarction. Int.J.Cardiol. 1998;67:219-224.

208. Lacy F, O'Connor DT, Schmid-Schonbein GW. Plasma hydrogen peroxide production in hypertensives and normotensive subjects at genetic risk of hypertension. Journal of Hypertension 1998;16:291-303.

209. Schroder E, Eaton P. Hydrogen peroxide as an endogenous mediator and exogenous tool in cardiovascular research: issues and considerations. Curr.Opin.Pharmacol. 2008;8:153-159.

210. Hu Z, Chen J, Wei Q, Xia Y. Bidirectional actions of hydrogen peroxide on endothelial nitric-oxide synthase phosphorylation and function: co-commitment and interplay of Akt and AMPK. J Biol Chem. 2008;283:25256-25263.

211. Sun J, Druhan LJ, Zweier JL. Reactive oxygen and nitrogen species regulate inducible nitric oxide synthase function shifting the balance of nitric oxide and superoxide production. Arch.Biochem.Biophys. 2010;494:130-137.

212. Boulden BM, Widder JD, Allen JC et al. Early determinants of H2O2-induced endothelial dysfunction. Free Radic.Biol Med 2006;41:810-817.

213. Thomas SR, Chen K, Keaney JF, Jr. Hydrogen peroxide activates endothelial nitric-oxide synthase through coordinated phosphorylation and dephosphorylation via a phosphoinositide 3-kinase-dependent signaling pathway. J Biol Chem. 2002;277:6017-6024.

214. Six I, Kureishi Y, Luo Z, Walsh K. Akt signaling mediates VEGF/VPF vascular permeability in vivo. FEBS Lett. 2002;532:67-69.

215. Gratton JP, Morales-Ruiz M, Kureishi Y et al. Akt down-regulation of p38 signaling provides a novel mechanism of vascular endothelial growth factor-mediated cytoprotection in endothelial cells. J Biol Chem. 2001;276:30359-30365.

216. Thomas SR, Chen K, Keaney JF, Jr. Hydrogen peroxide activates endothelial nitric-oxide synthase through coordinated phosphorylation and dephosphorylation via a phosphoinositide 3-kinase-dependent signaling pathway. J Biol Chem. 2002;277:6017-6024.

217. Aoyagi M, Arvai AS, Tainer JA, Getzoff ED. Structural basis for endothelial nitric oxide synthase binding to calmodulin. EMBO J 2003;22:766-775.

218. Greif DM, Sacks DB, Michel T. Calmodulin phosphorylation and modulation of endothelial nitric oxide synthase catalysis. Proc.Natl.Acad.Sci.U.S A 2004;101:1165-1170.

219. Reihill JA, Ewart MA, Hardie DG, Salt IP. AMP-activated protein kinase mediates VEGF-stimulated endothelial NO production. Biochem.Biophys.Res Commun. 2007;354:1084-1088.

220. Davis ME, Cai H, Drummond GR, Harrison DG. Shear stress regulates endothelial nitric oxide synthase expression through c-Src by divergent signaling pathways. Circ Res 2001;89:1073-1080.

221. Davis ME, Cai H, McCann L, Fukai T, Harrison DG. Role of c-Src in regulation of endothelial nitric oxide synthase expression during exercise training. American Journal of Physiology-Heart and Circulatory Physiology 2003;284:H1449-H1453.

222. Rush JWE, Denniss SG, Graham DA. Vascular nitric oxide and oxidative stress: Determinants of endothelial adaptations to cardiovascular disease and to physical activity. Canadian Journal of Applied Physiology-Revue Canadienne de Physiologie Appliquee 2005;30:442-474.

223. Sessa WC, Pritchard K, Seyedi N, Wang J, Hintze TH. Chronic exercise in dogs increases coronary vascular nitric oxide production and endothelial cell nitric oxide synthase gene expression. Circ Res 1994;74:349-353.

224. Boo YC, Sorescu G, Boyd N et al. Shear stress stimulates phosphorylation of endothelial nitric-oxide synthase at Ser1179 by Akt-independent mechanisms: role of protein kinase A. J Biol Chem. 2002;277:3388-3396.

225. Dimmeler S, Hermann C, Galle J, Zeiher AM. Upregulation of superoxide dismutase and nitric oxide synthase mediates the apoptosis-suppressive effects of shear stress on endothelial cells. Arterioscler Thromb Vasc Biol 1999;19:656-664.

226. Fukai T, Siegfried MR, Ushio-Fukai M et al. Regulation of the vascular extracellular superoxide dismutase by nitric oxide and exercise training. J.Clin.Invest 2000;105:1631-1639.

227. Wasserman SM, Mehraban F, Komuves LG et al. Gene expression profile of human endothelial cells exposed to sustained fluid shear stress. Physiol Genomics 2002;12:13-23.

228. Chen XL, Varner SE, Rao AS et al. Laminar flow induction of antioxidant response element-mediated genes in endothelial cells. A novel anti-inflammatory mechanism. J Biol Chem. 2003;278:703-711.

229. Duerrschmidt N, Stielow C, Muller G, Pagano PJ, Morawietz H. NO-mediated regulation of NAD(P)H oxidase by laminar shear stress in human endothelial cells. J.Physiol 2006;576:557-567.

230. Inoue N, Ramasamy S, Fukai T, Nerem RM, Harrison DG. Shear stress modulates expression of Cu/Zn superoxide dismutase in human aortic endothelial gels. Circ Res 1996;79:32-37.

231. Mowbray AL, Kang DH, Rhee SG, Kang SW, Jo H. Laminar shear stress up-regulates peroxiredoxins (PRX) in endothelial cells: PRX 1 as a mechanosensitive antioxidant. J Biol Chem. 2008;283:1622-1627.

232. Inoue N, Ramasamy S, Fukai T, Nerem RM, Harrison DG. Shear stress modulates expression of Cu/Zn superoxide dismutase in human aortic endothelial gels. Circ Res 1996;79:32-37.

233. Mowbray AL, Kang DH, Rhee SG, Kang SW, Jo H. Laminar shear stress up-regulates peroxiredoxins (PRX) in endothelial cells: PRX 1 as a mechanosensitive antioxidant. J Biol Chem. 2008;283:1622-1627.

234. Ookawara T, Haga S, Ha S et al. Effects of endurance training on three superoxide dismutase isoenzymes in human plasma. Free Radic.Res 2003;37:713-719.

235. Whyte JJ, Laughlin MH. The effects of acute and chronic exercise on the vasculature. Acta Physiol (Oxf) 2010;199:441-450.

236. Schafer G, Cramer T, Suske G et al. Oxidative stress regulates vascular endothelial growth factor-A gene transcription through Sp1- and Sp3-dependent activation of two proximal GC-rich promoter elements. J.Biol Chem. 2003;278:8190-8198.

APPENDIX A

INSTITUTIONAL REVIEW BOARD CONSENT FORM

Project Title:	**Genetics of In Vivo and In Vitro Endothelial Function in African Americans**
IRB Protocol #:	10831
Participant's Name and ID#:	

Investigators:

Michael D. Brown, Ph.D.
Associate Professor
Temple University
College of Health Professions
Department of Kinesiology

Deborah L. Crabbe, MD
Associate Professor, Medicine
Section of Cardiology
Cardiovascular Research Center
Temple University School of Medicine

M. Abul Kashem, MD, PhD
Section of Cardiology
Cardiovascular Research Center
Temple University School of Medicine

This study is funded by the National Heart, Lung, and Blood Institute of the National Institutes of Health.

1. PURPOSE OF THE STUDY

African Americans have hypertension more often than any other population in the United States. Most of the time, African Americans get hypertension at an earlier age and it causes more damage. Changes that happen to the blood vessels (the hollow tubes that carry blood through the body) may help to explain how a person gets high blood pressure. It is also known that a person's genetic make-up can play a role in getting hypertension. In most people, exercise can help to make these damaged blood vessels better, but a person's genetic make-up may affect how well exercise works for them.

You are being asked to join this study because you are between 40-75 years old and have a blood pressure between 120/80 and 159/99.

This is a research study and the purpose of the study is to understand how aerobic exercise and genes affect your blood pressure and blood vessels. Examples of aerobic exercise are fast walking, bicycling, and stair stepping.

2. DESCRIPTION OF THE PROJECT

If you qualify for the study, you will be enrolled for a total of 9-10 months. This includes a screening process, a diet, exercising, and testing before and after the exercise program. You will be one of many people participating in this study at Temple University.

This is not a weight loss study. In fact, the investigators want you to keep your body weight about the same during the study so that they can only look at the effects of exercise on your blood pressure and blood vessels. If you are a woman and taking hormone replacement medication for menopause, then you will continue your usual medication as prescribed by your doctor. A table showing the visits you will make and the amount of time needed for each visit is shown on the last page of this consent form.

Screening

You will have two or three separate screening visits to Dr. Brown's laboratory in the Department of Kinesiology at Temple University.

The first screening visit will take place in the morning after you have not eaten for 12 hours. Once you arrive to the laboratory, the staff will review with you what you will be doing on this first visit. First, you will give a urine sample for testing and then you will have your weight, height, and blood pressure measured. To give the urine sample, the staff will give you the appropriate items depending on if you are a man or a woman which you will take to the restroom next to the laboratory. You will collect some of your urine in the plastic container and return to the laboratory. The staff will then take the container of urine and dispose of the urine collection containers. Next, you will sit quietly for 15 minutes and then your blood pressure will be measured. You will then have a blood sample taken from your arm by staff trained in the procedure. The staff member will tighten a band around your upper arm, wipe your arm with alcohol and then insert a small needle in a vein in your arm. Three tubes of blood will be filled. One tube will be used to measure chemicals in your blood like glucose and salt in order get information about your health. The second tube of blood will be used to measure cholesterol and fat levels and the third tube will be for getting your DNA (Genetic material). The total amount of blood that will be taken is about 1½ tablespoons. The total time for this visit is approximately 1 hour.

It is possible that some of your DNA will also be frozen for future studies. However, this can only be done if you sign a separate consent form indicating that the

investigators can store a sample of your DNA for future use. If you decline to give consent for storage and future use of you samples, this will not affect your participation in the study.

If you are not using any medicine to lower your blood pressure, and your blood pressure is between 120/80 and 159/99, then you will qualify for the next screening visit. If you are using only one medicine to lower your blood pressure, then your blood pressure must be less than 130/85 in order to slowly stop your medication. If it is higher than this, then you will not be allowed to participate in the study. The study physician, Dr. Crabbe, will watch over the stopping of your medicine. Before your medication is slowly tapered, you will visit Dr. Brown's laboratory to get a small blood pressure machine and to go over the plan for stopping your medicine. During the time that your medication is being stopped, you must check your blood pressure every day and keep a log of the blood pressure values. You will also be given information telling you how to safely stop your medication. During the time that your medication is being stopped, you will begin an American Heart Association diet (see below). If your systolic blood pressure (top number) goes to 160 mmHg or your diastolic blood pressure (lower number) goes to 100 mmHg, then you will immediately contact the investigators. If you must restart your blood pressure medication you cannot take part in this study. If this happens, a letter will be sent to your personal physician explaining that you should start your usual treatment for your blood pressure. Four weeks after your blood pressure medication has been stopped, you will visit the laboratory in the morning for a second screening visit. During this visit, you will have your blood pressure measured. If your systolic blood pressure is between 120 and 159 and your diastolic blood pressure is between 80 and 99, while you are not taking blood pressure medication, then you will qualify for the next phase of the screening.

During the second screening visit you will have a physical examination by Dr. Crabbe, an ECG (a way for the doctors to look at how your heart functions to see if it is healthy) and have your blood pressure measured after 15 min of seated quiet rest. In order to have this test, a technician will apply small sticky pads to the skin of your upper body. At the location where the sticky pads are placed, your skin will be rubbed with an alcohol pad. Next, you will have an exercise test to see if you have any signs of heart disease. This test will be performed so that the investigators can be sure that the exercise program will be safe for your heart. During the exercise test, you will ride a bicycle and have pictures of your heart taken by echocardiography, sometimes called cardiac ultrasound. Echocardiography is one of the most commonly used tests for heart disease. It is non-invasive and involves placing a small wand on your chest. It uses sound waves to takes pictures of the heart. The test will take place at the Cardiovascular Center in Temple University Hospital. The test will begin easy and the pedaling will get harder every three minutes. The total time for the bicycle is approximately 8-12 minutes. You can ask the technician to stop the test at any time if you become uncomfortable. During this exercise test, your blood pressure and heart will be monitored. At certain times during the test, a technician will ask you to point to a chart to indicate how difficult the exercise is feeling. A physician will be present during the test. You understand that, if the test shows that you might have heart disease you will be excluded from the study at this point and you will be asked to be seen by your personal doctor or arrangements will be made for you to be seen by a doctor at Temple University Hospital. The total amount of time for this visit is 1 hour.

Baseline testing

Diet Program: After the second screening visit, you will go to a dietary class once per week for 6 weeks to learn how to eat an American Heart Association (AHA) Diet. This diet is called a "Step 1" diet because it is the first step in eating foods that are healthy for your heart. At each diet class, your weight and blood pressure will be measured. If the diet is causing you to lose weight, you will be asked to increase your intake of healthy foods slightly. The staff will help you figure out ways to do this. The amount of salt in your diet will be measured at the end of the 1 month period by providing another urine sample.

Submaximal VO_2 test: VO_2 stands for the amount of oxygen that your body uses when you are resting or doing physical work. Before the test begins, you will have your resting metabolic rate (A measure of how many calories your body burns) measured during 20 minutes of quiet rest while lying down on a table. VO_2 will be measured continuously during the 20 minutes by placing a hard plastic covering around your head for 20 minutes. You will just relax and breathe normally. After 20 minutes of quiet breathing, you will be prepared for the exercise test. The investigators need to measure your VO_2 during exercise in order to plan your exercise program. During this test, you will walk on a treadmill and wear a clip on you nose and have a tube connected to a mouthpiece so that the air you breathe out during the test will go into a machine that will measure oxygen and carbon dioxide. This test will start at a medium walking peed and the hill of the treadmill will get steeper and the walking speed will get a little faster every 3 minutes. Your blood pressure, heart rate, and your heart tracing (ECG) will be monitored before, during, and after the treadmill test. The test will be stopped when you reach 75% of your maximal exercise capacity. You will have this test three times, once before starting the exercise program and after 3 and 6 months of being in the exercise training program. The total amount of time that you will be on the treadmill is 8-12 minutes. The total amount of time for the visit is about 1 hour.

Ambulatory Blood Pressure Monitoring and Urine collection: Ambulatory blood pressure is the blood pressure in your body as you go about your regular day. On a separate day, you will begin a 24-hour blood pressure monitoring and urine collection period. This will happen on a day in which you have a normal schedule. You will visit Dr. Brown's laboratory in the morning between 7:00 AM and 9:00 AM. Laboratory staff will give you all of the materials required to complete the 24-hour period. The urine collection period will begin immediately. You will be fitted with a blood pressure monitor that will measure your blood pressure during the next 24 hours. The blood pressure monitor is a small electronic device that can go under your clothes. The monitor is connected to a blood pressure cuff that goes around your upper arm just like when you have your blood pressure measured. The blood pressure monitor will measure your blood pressure every 30 minutes during your waking hours and every 60 minutes during your sleeping hours. You will have the monitor for 24 hours so this means that you will have it when you go home and even when you go to bed. You will be asked to not exercise before or during the day of blood pressure monitoring. This means that you will not do any exercise or other physical activities that you would not regularly do. If you are walking about at the time of a blood pressure measurement, then you will stop if it is safe and pause until the measurement is completed. For example, if you are walking across the street and the machine begins to measure your blood pressure, you should continue across the street and then find a place to stop for a few minutes. You will be given a log book so that you

can write down what you are doing each time that your blood pressure is measured. You will be instructed to not remove the monitor except for bathing purposes, after which you will put the blood pressure monitor and cuff back on. Staff will show you how to take off and put on the blood pressure monitor and cuff. You will also be given the materials in order save all of your urine during the 24-hour period. 24-hours from the start of the blood pressure monitoring period you will give your last urine sample and remove the blood pressure cuff and turn off the monitor. This will end the 24-hour period. You will do have this test two times, once before and once after 6 the month exercise program.

Body composition and blood drawing: On the same day as the 24-hour ambulatory blood pressure monitoring and urine collection period, you will have your body composition (the amount of fat muscle and bone) measured. This measurement will tell the investigators what percentage of your body is fat. The instrument that measures your body composition is called bioelectrical impedance (BIA). The machine will cause a very small electrical current to go through your body for 2-3 seconds. It is one of the most common ways to measure your body composition. People who join a gym to workout often have this done at the gym before they start their exercise program. To do this test, you will lie on a table on your back with your left foot exposed. You will have to take off your left shoe and sock or remove any stockings. A technician will place two sticky pads on your left foot and two sticky pads on your left hand. The day before this test, you will be told to not exercise, drink alcohol, or eat food that is more salty than what you eat in your regular diet. This will help the investigators and you to get the most accurate information.

After your body composition is measured you will have blood samples taken so that the investigators can measure how you body changes with exercise training. This will be done twice during the study; once before and once after the exercise training. The blood will be taken the same way a described above in the screening visit. A needle will be placed in your arm vein and 6 tubes of blood will be obtained. These blood samples will be used to measure chemicals in your blood that help the investigators to know more about your blood vessels and blood pressure. Approximately 1 ounce (2½ tablespoonfuls) of blood will be taken. You will have your body composition measured two times, once before and once after the month exercise program. You will have your blood taken three times, once before, mid-way through, and at the end of the 6 month exercise program.

Blood Vessel Function Testing: The blood vessels are the small hollow tubes that carry blood through your body. They are called arteries and veins. This test will be done at the Cardiology Section at Temple University Hospital after an overnight fast (12 hours) so that the investigators can measure how well the blood vessels in your arm work. The investigators use an ultrasound machine to take pictures of a blood vessel in your arm. If you are right-handed, the test will be done on your left arm. If you are left-handed then the test will be done on your right arm. You will be asked to not eat or drink food or liquid that has caffeine, alcohol, or pain medicines like aspirin, Advil, or Motrin, and not take any decongestants, cold or allergy medicines for the whole day before the study. You will lie down comfortably on a table. Following 20 minutes of quiet rest on the table, a blood sample (about 1½ tablespoons) will be taken. First, the doctor will put a gel (Similar to Vaseline) on your arm. The doctor will place a small device called a wand on your skin near your elbow and hold it still for several minutes while pictures are being taken.

Next, the same measurement will be made, but this time, it will happen after 5 minutes of stopping the blood flow going into your arm. To do this, the doctor will put a cuff around your arm. The cuff is just like the cuff that is put on your arm to measure your blood pressure. Just like when your blood pressure is measured, the cuff is pumped up until the blood stops going into your arm. This test is the same except that the cuff will stay pumped up for 5 minutes. Your hand may begin to feel "numb and tingly" similar to the feeling when your hand or foot falls asleep. When the air is let out of the cuff, the measurements with the ultrasound machine will be made for three minutes. During this time you will continue to lie down on the table in a comfortable position.

After a 10-15 minute rest period, the same test will be done again but this time it will be done after small amount of a substance called a nitroglycerine tablet is placed under your tongue. Nitroglycerine is a substance that causes your blood vessels to relax. It is most often used when people have chest pain due to heart disease. Nitroglycerine can also lower your blood pressure for a short time. Very rarely, it causes a mild headache that last for 5-10 minutes.

During the same visit, two blood vessels in your neck (carotid arteries) will be measured to find out the thickness of the blood vessel walls. The thickness of the blood vessel walls in your neck is sometimes related to the risk for cardiovascular disease. This test will be done using the same ultrasound machine that was used to measure the blood vessel in your arm. The doctor will place a small amount of gel on each side of your neck and then place a small wand on the skin. Pictures will be taken for 3-5 minutes. The total time for this visit to measure arm and neck blood vessels is approximately 1 ½ hours. You will have this test done two times during the study; once before and once after the exercise training.

On a separate day, you will visit Dr. Brown's laboratory in the Department of Kinesiology at Temple University to have your blood vessels measured using a different kind of machine. For this test, you will also lie down comfortably on a table after not eating for 12 hours. You should not eat foods or liquids that have caffeine or alcohol in them and you will be told not to take an pain relievers, decongestants, cold or allergy medicines for the whole day before the test. Measurements will be made after 20 minutes of quiet rest. During the rest time, the investigators will comfortably support your arm in an armrest and put a blood pressure cuff on your upper arm. A second smaller blood pressure cuff will be put around your wrist. Next, a very thin hollow rubber band filled with mercury, called a strain gauge will be placed around your forearm. The test will begin when the investigators pump up the cuff around your wrist. Your hand will start to fell numb. The cuff around your upper arm will then be pumped up only a little bit every 15 seconds. During this time, blood pressure will be measured in your other arm. After these measurements and a 15-minute rest period, the investigators will again do the test but this time it will be after 5 minutes of having the cuff inflated just like what was done in the other test. This is when the cuff on your arm is pumped up very high for 5 minutes. After the 5 minutes, the air is let out of the cuff and the measurements will begin again and last for 3 minutes. This entire visit will last approximately 1 hour. You will have this test done two times during the study; once before and once after the exercise training.

Exercise Training Program

After completing the Baseline Testing described above, you will begin an aerobic exercise training program for 6 months. Aerobic exercise is physical exercise that uses large muscles like the legs and is continuous meaning is done for 20 minutes or more. It is not exercise like lifting weights. Aerobic exercise is the kind of exercise that doctors

say will help to lower blood pressure, lower cholesterol levels, and lower the chances of getting diabetes. Examples of aerobic exercise are fast walking and bicycling. You will visit the exercise facility in the Department of Kinesiology at Temple University 3 times per week. Study personnel will supervise all exercise sessions. You will learn how to measure your heart rate and to use heart rate monitors so that you will know how hard you are exercising. At your first exercise session, you will exercise for 15-20 minutes at the lowest level of difficulty. As you get in better shape, the amount of exercise you do will increase gradually until you are exercising for 40 minutes of moderate intensity exercise every session. The investigators do not want you to exercise as hard as you can because they know that lower levels of exercise are most healthy, They call this level of exercise "moderate intensity". You will be able to choose from different exercise machines. Exercise sessions will last between 40 and 60 minutes.

Final Testing

After you finish the 6 month exercise program, you will have everything re-tested in the same order as the testing that occurred during Baseline Testing. In addition, you will have the treadmill exercise test to measure your fitness level after the exercise training program. These final tests will happen 36-48 hours after one of your regular exercise sessions.

The total number of times that you will be stuck with a needle during the entire study is 4 (once during screening, once during baseline testing, one mid-way through the exercise program, and once during final testing). The total amount of blood that will be taken from your arm during the entire study is about 12 tablespoons over the 9-10 month period that you participate in the study.

Possible risks related to participation in this research study

The following risks, although low, are related to your participation in this research study.

Exercise testing: During the study, there are times when you will do a treadmill test that requires you to exercise as hard as you can. These tests are called maximal exercise tests. This is not the same as the exercise training in which you exercise 3 times a week. The risk of a maximal exercise test is that out of 10,000 tests, someone has a medical problem. In 1 out of every 70,000 exercise tests, a person will die from heart problems. In medical terms, doctors call this a rare event. The investigators will make sure it is as safe as possible for you to do this test because you will already have had tests including blood tests and a physical examination that will help the doctor to find out whether you are healthy enough to perform maximal exercise. Also, a doctor will be present when you do the test.

Giving blood: The research staff will take your blood in exactly the same way as when you have your blood taken at the doctor's office. There is a small risk of bruising and rarely infection. These risks will be lowered by using sterile procedures and by having trained research staff take all blood samples. There is also some pain associated with needle sticks and sometimes, people have been known to faint during needle sticks and blood drawing. We will take your blood while you are lying down which helps to prevent fainting.

Stopping your blood pressure medicine: The risks are that your blood pressure could increase to unsafe levels (greater than 180/120). Unsafe levels of blood pressure

can lead to headache, stroke, chest pain, heart attack and damage organs such as the kidneys and heart. These types of very high blood pressure emergencies are rare. Many doctors that treat high blood pressure feel that it is a good idea to reduce medicine once a year to see if the amount of medicine can be lowered. The investigators will only talk to you about stopping your medication if your blood pressure is not higher than 130/85 while you are taking your medicine. Your risk will be reduced because during this time you will also be changing your diet which may help to lower your blood pressure. In addition, the study doctor will check you as you begin to slowly stop your medicine. In order to help the study doctor make sure it is safe for you to stop your blood pressure medicine, the investigators will give you a blood pressure monitor to take home. The investigators will show you how to measure your blood pressure during the day. You will keep a log of your blood pressure numbers and report it to the investigators. If your blood pressure increases to more than 160/100, then the investigators will tell you to resume your medicine.

Measuring your body composition: There are no known risks of having the amount of fat measured in your body. There are no needles and no pain. Sticky pads are placed on your foot and hand. The test takes about 5 minutes.

Measuring Blood Vessel Function: The blood vessels are the small hollow tubes that carry the blood in your body. The risk of these tests is the minor discomfort you will feel when the blood pressure cuff is pumped up because it will cause the blood to stop going into your arm and hand and this will happen for 5 minutes. There are no procedures to lower the chances of having this discomfort. This discomfort is the same as when your foot falls asleep. There are no known risks of having ultrasound. During part of the test, a small nitroglycerine tablet will be placed under your tongue. Nitroglycerine can sometimes lower your blood pressure and sometimes cause a headache for 5-10 minutes. Your blood pressure will be prevented from going lower because you will be lying on a table. A Cardiologist will be performing the test and will monitor you during the entire visit.

Measuring your ambulatory blood pressure: You will be wearing a small device that will measure your blood pressure during a regular day. When the blood pressure monitor pumps up the cuff, it is possible to hear the sound of the pump when you are in a quiet place. About 2 out of 100 people say that they have woken up during the night. These people also say that they are light sleepers. At night, the machine will measure your blood pressure 1 time every hour. There are no procedures to lower the chances that the blood pressure machine might wake you while you are sleeping. The investigators will show you ways that might help so that this does not happen.

Exercise training: The risk of exercise training is that it is possible to have a medical problem usually related to your heart. Out of every 375,000 hours of exercise training there are 2 times in which a person has a medical problem. This is the same as 1 medical problem for every 1.7 million miles of walking. These risks will be lowered because you will have a physical examination and an exercise test to make sure it is safe for you to train. There will also be trained staff that knows how to handle medical problems if it happens during an exercise training session.

Genetic Testing: As part of the study, the investigators will be analyzing your DNA to see if it gives them information about how your blood vessels work and how your blood vessels and blood pressure are affected by exercise. DNA is the material in your body that is passed on from parent to child and from generation to generation. The investigators will get your DNA during one of the times that they take your blood at the start of the study. The risks of having your blood taken have already been described

above. The risk of genetics testing is finding out that you have a gene that shows that you may have a higher risk for getting a disease in the future. These risks are low because the places in your DNA that the investigators are looking at do not tell them if you will or will not get cardiovascular disease in the future.

Since there may be unknown risks to pregnant women and their unborn child, if you are nursing, pregnant, or planning to become pregnant, you will not be allowed to participate in this research study.

You confirm to the best of your knowledge that you are not pregnant and if you become pregnant during the course of this study, you must notify your physician and the investigators immediately.

Possible benefits of participating in this study

It is well known that African Americans suffer more from high blood pressure (hypertension) compared to other populations in the United States. There are direct benefits to you as a result of your participation in this study. Some of these benefits are greater than those you would have from usual medical testing. For example, 24-hour ambulatory blood pressure monitoring, dietary counseling, exercise testing, cardiac ultrasound, and supervised exercise training are not usual medical practice procedures. You will benefit from the medical and cardiovascular testing, measurement of your cholesterol and glucose. Most experts think that exercise is usually good for your overall health. The benefits of aerobic exercise training on risk factors for cardiovascular disease are well known. When blood pressure is lowered, it lowers your chances of getting heart disease and having a stroke. Even when blood pressure is not lowered with exercise training, healthy changes in body composition, cholesterol, and glucose and insulin almost always happen. You will also benefit from the diet. This diet is the first step to a low fat/low salt diet that is healthy for your heart. The benefits of a lower fat and salt diet are also well known. It is the investigator's hope that the exercise becomes an enjoyable experience and that you will enjoy exercising with others who share many of the same health and fitness goals as you do. The benefits of dietary counseling and exercise training have been shown in large studies involving many participants. Whether these benefits will occur in you cannot be guaranteed.

Alternative Treatments

Alternative treatments to aerobic exercise training are very limited. Of course, under your physician's direction, there is the option of increasing your medications to control your blood pressure. This may be the case even if exercise does lower your blood pressure. However, blood pressure medicine cannot do all of the things that aerobic exercise can. All of the side effects of aerobic exercise training in terms of health are beneficial. There are other treatments that do not use medication. Lowering the amount of salt in your diet and reducing your body weight if you are overweight may help to lower your blood pressure too. As with exercise, these treatments may not be effective for every person, and, each person may respond differently to them. You should always ask your doctor before you start any of these ways to help treat your blood pressure. You also have the choice to not participate in this study.

Confidentiality Statement

All documents and information about to this study will be kept confidential in accordance with federal, state, and local laws and regulations. You understand that

medical records and data generated by the study may be reviewed by Temple University's Institutional Review Board, the Office for Human Research Protections, and the National Institutes of Health to assure proper conduct of the study and compliance with federal regulations. You understand that the results of this study may be published. If results are published, you will not be identified by name.

Voluntary Participation Statement

You understand that participation in this study is entirely voluntary, and that refusal to participate will involve no penalty or loss of benefits to you. You may discontinue your participation at any time without penalty or loss of benefits.

Compensation Statement

You understand that you will receive $150 if you complete this study and attend at least 90% of the exercise training sessions. You understand that you will receive $50 if you complete the baseline testing, an additional $50 if you complete the exercise training with at least 90% attendance, and an additional $50 if you complete the final testing. You will receive compensation for your participation in the form of cash at the end of the study. If you do not complete the entire study you will receive partial compensation for those parts of the study you do complete.

Institutional Contact

If you have questions about your rights as a research participant, you may contact the Institutional Review Board Coordinator at (215) 707-3390

If you have questions about research-related injuries, you may contact the Principal Investigator, Dr. Michael Brown, in the Department of Kinesiology at (215) 204-5218.

Standard Injury Statement

You understand that if you sustain an injury as a result of participation in this study, the physician's fees and medical expenses that result will be billed to your insurance company or you in the usual manner. You understand that financial compensation for such injuries is not available. You understand that you have not waived any legal rights that you would otherwise have as a participant in an investigational study.

Costs Statement

You understand that any doctor's fees, medical tests, or other tests associated with this study will be provided at no cost to you. You understand that you are responsible for transportation to the study site and parking.

Termination Statement

The investigators have the right to terminate your participation without regard to the your consent. This could occur if you cannot make your appointments, miss more than 10% of your exercise sessions, or experience a change in your medical condition during the course of the study.

Statement of Significant New Findings

You will be informed in a timely manner of any new information regarding this study that may have an affect on your willingness to participate, continue your participation, or

after your participation that may have an affect on your future medical care. You may be asked to sign a revised informed consent that contains this new information.

Final Statement and Signature

This study has been explained to me, I have read the consent form and I agree to participate. I have been given a copy of this consent form.

_____

Participant's signature Date

_____

Principal Investigator's signature Date

_____

Witness's signature Date

Timeline	Visit	Procedure	Required
Month 1	*Orientation Visit*	**1.** Review medical history questionnaire, informed consent **2.** Blood pressure taken.	1 hour time
Month 1	*Before Screening Visit 1*	12 hour overnight fast evening before screening visit 1	12 hours intake monitoring
Month 1	*Screening Visit 1*	**1.** Blood and urine sample drawn **2.** Blood pressure taken.	1 hour time
Month 1	*Screening Visit 2*	Physical exam and exercise stress echo test	1 ½ hours time
Month 2	*Dietary Stabilization Period*	**1.** Learn and maintain AHA diet. **2**. Complete food records. **3**. Meet 1 session/week for 6 weeks	**1.** Monitor and maintain dietary intake. **2.** Attend 2 dietary sessions a week for

			4 wks
Month 2	*Before Baseline Testing*	12 hour overnight fast evening before first visit.	12 hours intake monitoring
Month 2	*Baseline Testing*	**1.** Blood samples **2**. Body composition tested. **3.** Blood pressure taken. **4.** Blood vessel function tests **5.** 24 hour urine and BP collection. **6.** Submaximal treadmill test to measure fitness level	*Several visits:* **1.** 1 ½ hours: Collection of blood, urine, blood pressure. **2.** Body comp. taken and take home supplies for 24 hour collection. **3.** After 24 hour collection, drop off supplies and samples. 4. 1 ½ hours for blood vessel function testing
Months 3-8	*Exercise Training*	Supervised exercise training sessions:	3 sessions a week for 6 months
Month 9	*Before Final Testing*	12 hour overnight fast evening before first visit of final testing.	12 hours intake monitoring
Month 9	*Final Testing*	Repeat Baseline Testing	Same as baseline testing