Aerobic Exercise and Redox Modulation in the Adipose Microvasculature

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THESIS

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DEDICATION PAGE

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CONTRIBUTION OF AUTHORS

Chapter III contains data from a published manuscript and was used with permission from Wolters Kluwer Health Lippincott Williams & Wilkins© I was the first author. I assisted with study design, collected the data, analyzed the majority of the data, generated the figures, and wrote the manuscript. Nina C. Franklin helped design the study and edit the manuscript. Edita Norkeviciute assisted with training participants assigned to receive exercise training and with data analysis. Jing Tan Bian established lab protocols for collecting data from *ex vivo* microvessels and assisted with experimental design. James C. Babana and Mary R. Szczurek assisted with data collection and helped edit the manuscript. Shane A. Phillips helped design the study, edit the manuscript, and provided all materials.

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ABBREVIATIONS

ACE	Angiotensin converting enzyme
ACh	Acetylcholine
Akt	Protein Kinase B
AMPK 5'	Adenosine monophosphate-activated protein kinase
ANG I	Angiotensin I
ANG II	Angiotensin II
AT	Aerobic training
BH_4	Tetrahydropterin
BLP	Bilateral leg press
cGMP	Cyclic guanosine monophosphate
CON	Control group
CREB	Cyclic adenosine monophosphate response element-binding protein
CRP	C-reactive protein
CVD	Cardiovascular disease
DAG	Diacylglycerol
ECE1	Endothelin converting enzyme 1
ET1	Endothelin 1
FAD	Flavin adenine dinucleotide
FFA	Free fatty acid
FMD	Flow mediated dilation
FMN	Flavin mononucleotide
GPCR	G protein coupled receptor
HAMECs	Human adipose microvascular endothelial cells
HUVECs	Human umbilical vein endothelial cells
H_2O_2	Hydrogen peroxide
IP_3	Inositol 1,4,5-trisphosphate

ABBREVIATIONS (Continued)

Kir	Potassium inward rectifying channel
L-NAME	L-N^G-Nitroarginine methyl ester
L-NMMA	N^G-monomethyl-L-arginine
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate)
$NO^{\cdot}$	Nitric oxide
NOS	Nitric oxide synthase
NOX II	NADPH Oxidase
NTG	Nitroglycerin
PEG-Cat	Polyethylene glycol Catalase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PKG	Protein Kinase G
RAS	Renin Angiotensin System
ROS	Reactive oxygen species
O_2^-	Superoxide
SOD	Superoxide Dismutase
TNFα	Tumor necrosis factor-alpha
Zn-DDC	Zinc-diethyldithiocarbamate

Chapter I. Introduction

I.A Introduction to the Problem:

Cardiovascular disease (CVD) is the number one cause of death in the post industrial world [1-3]. Lifestyle management plays a large role in the prevention of CVD. A number of medical conditions that are risk factors for CVD including hypertension, hyperglycemia, dyslipidemia, and obesity are largely influenced by modifiable risk factors including cigarette smoking [4, 5], poor nutrition [6-8], and physical inactivity[9]. In fact, regular physical activity is associated with reduced risk of all-cause mortality, hypertension, stroke, metabolic syndrome, type II diabetes mellitus, certain forms of cancer, and depression [10-12]. Strenuous physical exertion during exercise and high amounts of moderate physical activity are also associated with longer life expectancy [13, 14]. This finding may be associated with previous observations indicating exercise can reverse age-related declines in artery health as measured by dilatory function [15]. Lastly, regular exercise is recommended for the prevention and management of obesity, another epidemic in the United States that is a major risk factor for CVD [16]. Obesity related inflammation may contribute to impaired adipose tissue blood flow which may further contributes to systemic inflammation and oxidative stress [17].

One of the primary mechanisms through which regular physical activity results in improved artery health may be through improved endothelium function or the restoration of endothelium health in diseased states [18]. This adaptation appears to be protective independent of reduction in traditional risk factors, as it has been found that regular exercise training (strenuous physical exertion) and high amounts of moderate levels of physical activity are ~40% more protective than would be predicted based on changes in traditional risk factors alone. [19] The mechanisms through which exercise improves the health of the endothelium are

not completely understood, however a reduction in oxidative stress does appear to play a role [20]. A transient increase in shear stress during exercise has been proposed as one of the primary mechanisms responsible for exercise training adaptations on the vasculature [21, 22]. In isolated endothelial cells high physiologic levels of shear stress have been shown to improve the redox environment [23]. Thus in conditions associated with increased oxidative stress, such as obesity, exercise may be of even greater importance.

Despite the long term benefits of regular physical activity, the potential harmful effects of acute bouts of exercise on the vasculature in sedentary populations are not as well studied, particularly in overweight and obese individuals. Acute strenuous physical exertion has been shown to paradoxically increase the risk of cardiovascular events in sedentary adults [24, 25]. Experimental studies also indicate clinical measures of arterial function such as brachial artery flow mediated dilation are impaired after acute bouts of strenuous exertion in sedentary, elderly and patient populations [26-30]. The increased risk of cardiovascular events associated with acute bouts of physical exertion is likely due to the fact that sedentary people are unaccustomed to exercise. Acute exercise alters hemodynamic responses in order to meet increased metabolic demand. These alterations in hemodynamics include an increase blood pressure and an increase in oxidative stress [31-34].

Interestingly physically active individuals are not at an increased risk of cardiovascular events during acute bouts of strenuous physical exertion and experimental studies show that exercise trained individuals do not experience a decrease in clinical measures of arterial function despite increases in blood pressure during exercise similar to sedentary populations. [27, 30, 34-38]. Isolated resistance arteries taken from sedentary individuals and resistance exercisers immediately before and after an acute bout of resistance exercise demonstrate that resistance

arteries from the regular exercisers buffer oxidative stress by dismutation of superoxide to hydrogen peroxide [36]. Hydrogen peroxide has been shown to maintain vasodilatory function in place of nitric oxide [36, 39, 40]. This study also found that isolated exposure to high pressure mimicked the effects of acute strenuous physical exertion. This latter finding further implies that high blood pressure exposure during exercise plays a critical role in elevated oxidative stress and vascular dysfunction.

The importance of studying the potential harmful effects of acute bouts of exercise on the vasculature in sedentary populations and the vascular adaptations that protect regularly physically active individuals against vascular dysfunction associated with acute bouts of exercise is several fold: (1) it may allow us to identify mechanisms contributing to this increased risk in cardiovascular events with acute exercise and thus prevent them via pretreatment with medications or supplements; (2) findings may enhance initial exercise prescription leading into a regular training program in sedentary populations (i.e. mode, intensity, duration); and (3) some of the proposed mechanisms through which acute bouts of exercise may contribute to an increase in cardiovascular events are characteristic of CVD risk factors (e.g. high blood pressure, increased free fatty acids). Thus we may be able to elucidate potential mechanisms through exercise protects against CVD more than would be predicted by management of traditional risk factors and better understand vascular adaptations that occur with exercise. Investigation of the potential harmful effects of acute bouts of exercise on the vasculature in obesity is of particular importance as there is an epidemic of obesity in the United States and this condition is associated with heightened oxidative stress and vascular dysfunction [17].

I.B Purpose of the study:

The purpose of the studies undertaken for this doctoral dissertation include: (1) to investigate if regular aerobic exercise training protects previously sedentary overweight and obese individuals from acute physical exertion induced vascular dysfunction; (2) based on the hypothesis that exposure to high blood pressure is one of the main mediators of acute physical exertion induced vascular dysfunction, we sought to further elucidate the mechanisms through which high pressure may cause vascular dysfunction; (3) determine mechanisms through which the regularly exercised vasculature is protected against high pressure induced vascular dysfunction; and (4) to determine if shear stress, which is thought to be one of the primary mediators of exercise induced vascular adaptation, promotes changes in endothelial cells that may protect the endothelium against exercise and high pressure induced dysfunction.

There is little published work on the effects of acute physical exertion on endothelium function in obesity [27-29, 35]. Furthermore there is no published literature on the role of regular aerobic exercise to prevent acute physical exertion induced vascular dysfunction in overweight and obese individuals. This population may be at heightened risk as obesity negatively alters the adipose tissue microenvironment and this may impact vascular function [17]. Previous findings suggest endothelium function is impaired in obese indivudals after high intensity aerobic exercise [38] and resistance exercise [27-29]. All of the studies indicating that regular exercisers (not necessarily overweight or obese) are protected against acute strenuous physical exertion induced vascular function were cross sectional in design [37, 38, 41]. Therefore, there was a need for an intervention study to definitively show that regular aerobic exercise protects against acute physical exertion vascular dysfunction. Furthermore the need remained to address the mechanisms through

which high pressure may cause vascular dysfunction and how regular exercise may buffer this vascular dysfunction.

The overall aim of this project was to (1) determine if regular aerobic exercise training prevents acute physical exertion-induced vascular dysfunction in sedentary, overweight and obese adults; and (2) determine if regular exercise promotes a favorable redox environment in the vasculature. The hypotheses were that regular aerobic exercise training prevents acute physical exertion-induced vascular dysfunction in sedentary, overweight and obese adults, and that high aerobic fitness confers the ability to buffer oxidative stress associated with acute exertion and high pressure.

I. C Specific Aims

Specific Aim I : (Ia) Determine if regular aerobic exercise training prevents acute physical exertion-induced arterial dysfunction in sedentary, overweight and obese adults. (Ib) Determine the mechanism through which regular aerobic exercise training prevents acute physical exertion-induced arterial dysfunction.

Hypothesis (Aim I): (Ia) Post-acute exertion-induced brachial artery and microvessel dysfunction would be prevented, and (Ib) hydrogen peroxide contributes to preserved microvessel vasodilator function following acute physical exertion in obese adults.

Specific Aim II: (IIa) Determine the mechanisms through which high pressure induces vascular dysfunction in *ex vivo* microvessels and (IIb) how exercise training may attenuate high pressure induced vascular dysfunction.

Hypothesis (Aim II): (IIa) High pressure induces results in excessive oxidative stress via activation of the local renin angiotensin system and (IIb) exercise protects against this cascade by decreasing pro-oxidant enzyme expression and increasing antioxidant enzyme expression.

Specific Aim III: (IIIa) Determine if Angiotensin II treatment increases oxidative stress in human adipose microvascular endothelial cells (HAMECs) and (IIIb) investigate the role of shear stress in modulating the redox environment in HAMECs.

Hypothesis (Aim III): (IIIa) Angiotensin II will elicit increased oxidative stress in HAMECs and this will be prevented by inhibitors of the renin angiotensin system and NADPH Oxidase II. (IIIb) Shear stress promotes a favorable redox balance in HAMECs similar to aerobic exercise.

Chapter II. Literature Review

II. A Vascular function and dysfunction

The endothelium controls vascular homeostasis maintaining balance between vasodilator and vasoconstrictor substances (autocoids) through the release of paracrine factors that inhibit platelet activation and aggregation, preventing leukocyte adhesion, and limit proliferation of vascular smooth muscle [42-44]. The endothelium's location at the interface between the blood and the blood vessel wall results in exposure to a vast number physical and chemical signals that must be transmitted for homeostasis. The primary autocoid responsible for these regulatory effects is nitric oxide ($NO^{\cdot}$), although prostacyclin, hydrogen peroxide (H_2O_2), and other undefined endothelium-derived relaxing factors (EDRF) play a role in this milieu as well. Endothelium dysfunction (paracrine dysfunction of the inner lining of blood vessels) is a systemic pathological state that precedes the development of atherosclerosis and CVD [45, 46]. Endothelium dysfunction also contributes to the pathogenesis of atherosclerosis by fostering vasospasm, vascular inflammation, thrombosis formation, platelet aggregation, and proliferation of vascular smooth muscle. In addition, endothelium dysfunction often occurs in conjunction with other CVD risk factors [43, 47].

With endothelium dysfunction preceding atherosclerosis, it is of little surprise that it is associated with a host of risk factors also associated with CVD including hypertension [48, 49], hypercholesterolemia [50, 51], and cigarette smoking [52, 53]. It is now known that the development of atherosclerosis can begin as early as childhood [45] and endothelium dysfunction has been detected in children with hypercholesterolemia [54, 55]. Furthermore, exercise has been shown to improve endothelium function in obese children [56].

Following its inception in seminal publications by Anderson and Mark [57] and Celermajer et al [55], flow-mediated dilation (FMD), a noninvasive assessment of endothelium function emerged as the most often used means of measuring endothelium function [55, 57]. This assessment includes measuring endothelium function using temporary arterial occlusion (generally five minutes) to induce a reactive hyperemia (supra-compensatory blood flow) when the occlusion is terminated and measurement of the subsequent dilation induced by the increase in flow. Some epidemiological studies have found FMD to add prognostic value, independent of traditional risk factors, in predicting future development of CVD or cardiovascular events [58, 59]. However the comparison of endothelium function, as measured via FMD between labs remains challenging. The methodology and analysis employed by different groups varies despite numerous methodological recommendations being published [60, 61].

Initially brachial artery FMD was thought to serve as a bioassay for ($NO^{\cdot}$). However a recent study demonstrated that inhibition of $NO^{\cdot}$ production via N^{G}-monomethyl-L-arginine L-(NMMA) did not significantly reduce FMD [62]. In addition multiple recent studies report that $NO^{\cdot}$ does not solely govern radial artery FMD [63-65]. Cuff placement of the occlusion cuff during the FMD procedure is also thought to alter the $NO^{\cdot}$ dependency of the subsequent FMD. Cuff placement distal to the imaged artery leads to a more $NO^{\cdot}$-dependent dilation compared to a proximal cuff placement [59]. Taken together these findings suggests that $NO^{\cdot}$ contribution to FMD is highly variable and contingent on various factors such as vessel size and the methodological approaches used when testing the FMD response. Other vasodilatory agents include potassium ions, prostacyclin, H_2O_2 and EDRF [39, 66]. Nonetheless, reduced $NO^{\cdot}$

bioavailability is thought to contribute to endothelium dysfunction and provide prognostic value for CVD and cardiovascular events [59].

Oxidative stress results from an imbalance in favor of pro-oxidant status over anti-oxidants and is thought to play a role in endothelium dysfunction [42, 43] Oxidative stress is associated with the development of CVD[67]. The primary mechanisms through which oxidative stress may contribute to vascular dysfunction is via a reduction in $NO^{\cdot}$ bioavailability by scavenging formed $NO^{\cdot}$ and reducing the capacity of nitric oxide synthase (NOS) to produce $NO^{\cdot}$ [68]. Endothelium dysfunction associated with CVD risk factors, such as hypercholesterolemia, hypertension, type II diabetes mellitus, and cigarette smoking is at least partially attributable to excess oxidative stress in the form of reactive oxygen species (ROS) primarily superoxide (O_2^-) and the subsequent decrease in $NO^{\cdot}$ [69]. Other metabolites associated with oxidative stress include H_2O_2 and hydroxyl radicals ($^{\cdot}OH$). Nitrogen species include peroxynitrite ($ONOO^-$) which is the product of O_2^- and $NO^{\cdot}$. These species do not impart equal damage throughout the vasculature, as they vary in respect to the reactions they facilitate, their reactivity, and half-life. For example, $^{\bullet}OH$ is of little relevance in terms of physiological signaling because it is a very unstable radical [70]. The mechanisms through which oxidative stress occurs and disrupts $NO^{\cdot}$ signaling will be elucidated below.

II. B Nitric oxide signaling

Endothelial Nitric oxide Synthase (eNOS aka NOS3) is a constitutively Ca^{2+} - dependent member of the NOS family of enzymes. The NOSs are complex enzymes that ultimately act as catalysts in the oxidation of the amino acid L-Arginine to L-Citrulline and gaseous $NO^{\cdot}$ under favorable physiological conditions [71]. The other two members of the NOS family include

neuronal NOS, or nNOS aka NOS1 and inducible NOS, or iNOS aka NOS2. The three isoforms share 50–60% homology at the amino acid level [71, 72]. eNOS derived $NO^{\cdot}$ is the predominate endogenous vasodilatory autocoid in humans [73]. In addition $NO^{\cdot}$ has anti-proliferative and anti-apoptotic properties which render protective against atherosclerosis. In addition NOS1 and NOS2 mediated $NO^{\cdot}$ also plays a role as a neurotransmitter, in insulin signaling, airway tone, and peristalsis. NOSs are very tightly regulated by five cofactors and several post-translational phosphorylation events on Serine (S), Threonine (T), and Tyrosine (Y) residues in addition to co- and post-translational lipid modifications [74, 75].

The five cofactors of eNOS are Flavin adenine dinucleotide (FAD), Flavin mononucleotide (FMN), heme, tetrahydrobiopterin (BH_4) and calmodulin [73]. The C-terminus of eNOS has a Nicotinamide adenine dinucleotide phosphate (NADPH) binding domain where reduction of NADPH occurs. This C- terminus reduction of NADPH transfers electrons to FAD, subsequently to FMN, and ultimately to the N-terminus oxidase domain, which contains a heme, and binding sites for L-Arginine, BH_4 and calmodulin. Elevated cytoplasmic Ca^{2+} levels activate calmodulin, which binds to the calmodulin binding domain in eNOS. Calmodulin binding subsequently promotes the alignment of the oxidase and reductase domains of eNOS, which is requisite for optimal $NO^{\cdot}$ synthesis. Functional eNOS occurs as a reciprocal homodimer [71, 73, 76]. In fact increased ratio of eNOS monomers to dimers has been found to contribute to vascular aging [77]. BH_4 deficiency is also frequently associated with impaired vascular function. Yang et al [77] found reduced levels of BH_4 via HPLC in mesenteric arteries from aged mice compared to young mice. Two molecules of BH_4 are bound to each eNOS dimer complex and facilitate electron transfer from L-Arginine to the intermediate N-hydroxy-L-Arginine and subsequently to L-Citrulline. BH_4 deficiency results from one of two means: 1) Decreased expression of GTP cyclohydrolase I and

sepiapterin reductase, the enzymes responsible for BH_4 synthesis; and 2) increased BH_4 oxidation in times of oxidative stress [77]. BH_4 deficiency ultimately results in eNOS uncoupling whereby eNOS produces O_2^- instead of $NO\cdot$ when the final electron transfer is to O_2 .

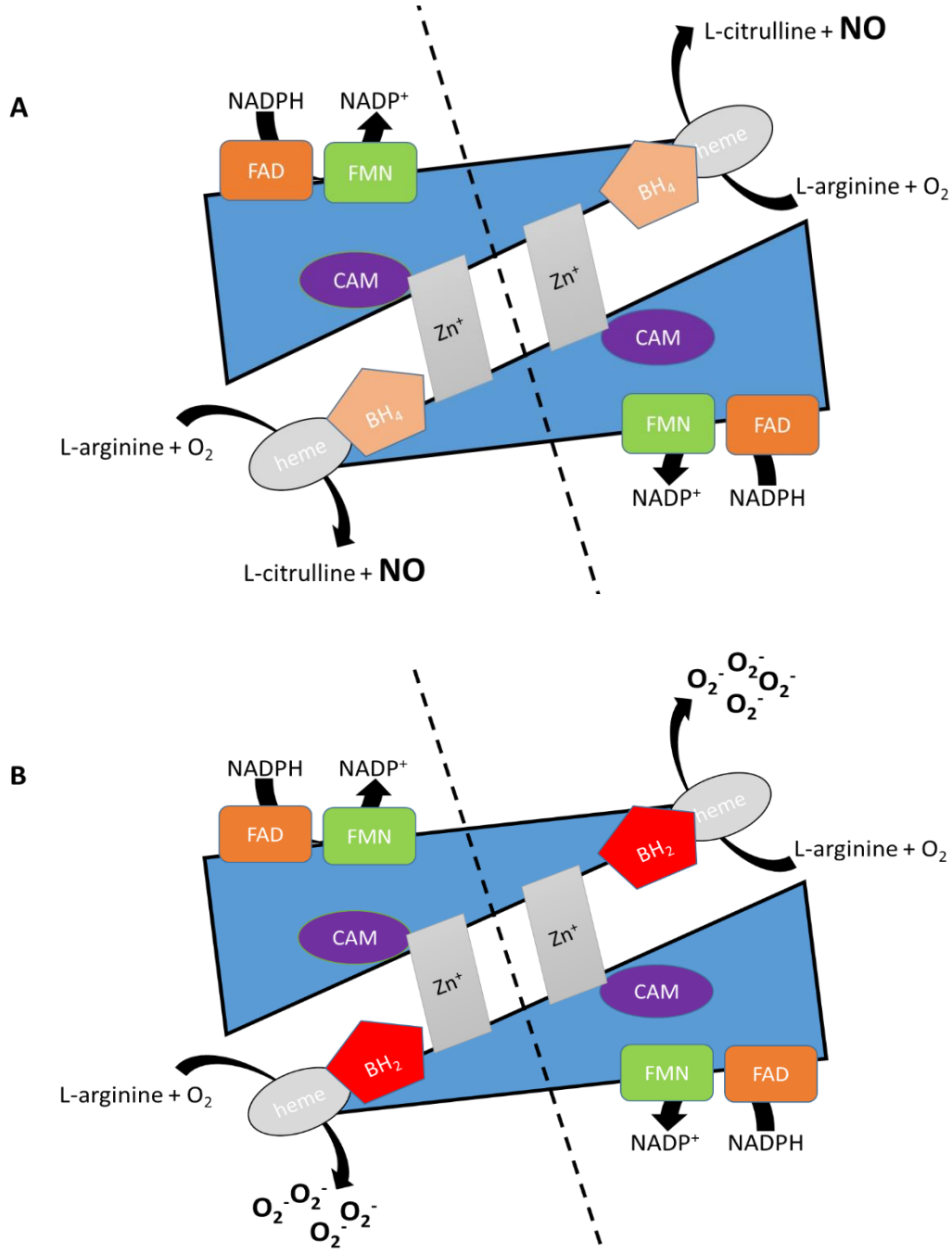

Figure 1 Illustration of coupled and uncoupled eNOS.

(A) Electron transfer commences at oxidation of NADPH via FAD and continues to FMN at the reductase domain. Electrons are then transferred to a heme ion in the oxygenase domain. BH_4 is important in the delivery of an electron and proton to intermediates of l-arginine to $NO^{\cdot}$. Calmodulin (CAM) facilitates electron transfer within the eNOS enzymatic complex. Zinc (Zn) ions are also an integral component of eNOS in regards to heterodimer formation and enzyme stability. **(B)** When BH_4 is oxidized to BH_2 or eNOS occurs as a monomer this reaction becomes uncoupled and eNOS produces O_2^-. Inspired by [78, 79]

II. B. I Covalent Nitric oxide Synthase modifications – phosphorylation

Shear stress and agonist mediated dilation (i.e. acetylcholine, insulin, bradykinin, etc) via $NO^{\cdot}$ mediated dilation are contingent upon phosphorylation of S residues [73, 74]. For example intracellular signaling kinases including protein kinase B (Akt), protein kinase A (PKA) and adenosine monophosphate-activated protein kinase (AMPK) are all phosphorylated in response to shear stress in cultured endothelial cells or isolated microvessels [80]. Multiple studies have found that all three of these kinases phosphorylate eNOS at S1177. Additionally the Akt pathway results in phosphorylation of eNOS at S617 and the PKA pathway results in phosphorylation of eNOS at S635 [73, 80-82]. In contrast, increased diacylglycerol (DAG) levels can activate PKC to phosphorylate T495, which is thought to negatively regulate eNOS mediated $NO^{\cdot}$ production [83].

II. B. II Covalent Nitric oxide Synthase modifications – acylation

Apart from post-translational phosphorylation events, eNOS is tightly regulated via co- and post-translational lipid-protein adduction, specifically myristoylation and palmitoylation. Myristoylation is a lipid modification whereby a myristic acid (14:0) derived myristoyl group is covalently bonded to an N-terminus Glycine (G) residue via an amide bond [84]. Palmitoylation, which is the most common acylation of proteins in eukaryotic cells [85] is the covalent attachment of palmitoyl group (16:0) bonded to a Cysteine (C) group via a thieoester bond. Palmitoylation also can occur with S and T residues but only the C residue palmitoylation appears to be of relevance in the context of eNOS signaling. An important distinction between myristoylation and palmitoylation is that myristoylation is irreversible and palmitoylation is reversible. Myristoylation occurs co-translationally, whereas palmitoylation is post-translational. Thus, myristolyation precedes palmitoylation and in some situations is requisite for subsequent

palmitoylation [85]. These lipid modifications play a role in regulating protein subcellular localization, membrane stability, intracellular trafficking, interaction with effectors and cell signaling. Sessa and colleagues contributed greatly to the seminal work characterizing the role of lipid modifications and its importance in eNOS intracellular localization and function. [73-75, 86-88]

II. B. III Subcellular Nitric oxide Synthase localization and Caveolae interaction

In endothelial cells, lipid modifications play a large role in targeting eNOS to the Golgi apparatus and caveolae. Caveloae are small invaginations of the plasma membrane, containing the caveolin family of protein (particularly Caveolin-1), cholesterol, sphingolipids, G-proteins coupled receptors (GPCRs), insulin and growth factor receptors, integrins, phosphatidylinositol 4,5-bisphosphate (PIP_2), and various actin binding proteins. Hence caveolae play a large role in various cellular signaling pathways, particularly in the endothelium [73, 85, 89]. eNOS is co-translationally myristoylated at the G2 residue of the N-terminus and subsequently palmitoylated on residues C15 and C26 [75, 87]. Hydrophobic amino acids flank these C residues ((Glycine-Leucine)$_5$ repeat) facilitating the palmitoylation. Liu et al [75] used molecular cloning techniques to manipulate eNOS DNA and mutate the five Leucines between residues C15 and C26 to Serines and found that palmitoylation no longer occurred while also verifying that actual protein content did not vary with this mutation. eNOS–green fluorescent protein (GFP) chimeras indicated that G2 myristoylation and C15/26 palmitoylation were essential for co-localization with the Golgi. In addition GFP, was fused at different amino acid residue segments along eNOS in order to determine which residues were obligatory for appropriate intracellular localization and it was found that the first 35 amino acids of eNOS are sufficient to target GFP into the Golgi region, which satisfies logic since the first 35 amino acid sequence includes the myristoylation site (G2)

and palmitoylation sites (C15 and C26) needed for optimal eNOS activation. Myristoylation and palmitoylation did not occur in a eNOS (1–35) GFP peptide without the G-L repeat. This finding indicates that residues following C26 are critical for recognition by palmitoyl transferase, the enzyme that catalyzes palmitoylation (see figure 2).

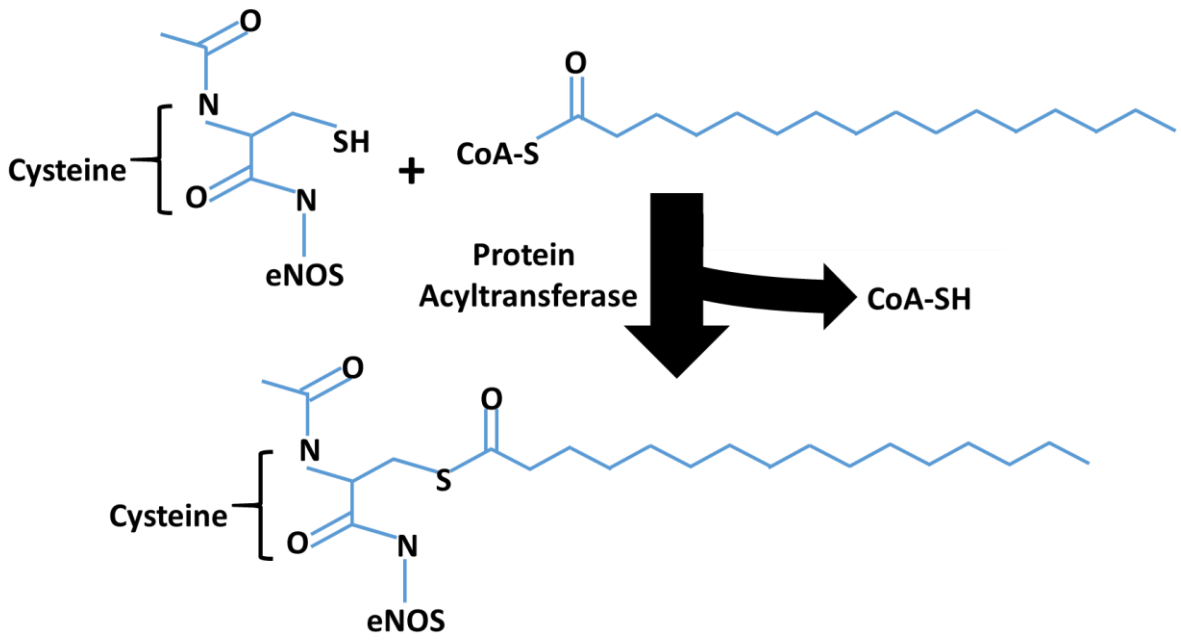

Figure 2. Acylation plays an important role in eNOS subcellular trafficking.

This illustration depicts palmitoylation of a Cysteine residue. In the case of eNOS this represents residue 15 or 26 and is essential for targeting eNOS to the golgi or caveolae at the cell membrane. Inspiration for this image comes from [90] and the AOCS Lipid library.

Palmitoylation is critical in trafficking eNOS to the plasma membrane [86-88]. Shaul et al [91] used pulmonary artery endothelial cells to measure eNOS content via immunoblotting in various fractions of the plasma membrane. eNOS protein was detected in caveolar membrane fractions but was not found in non-caveolar plasma membrane. eNOS activity was determined in the subcellular fractions of endothelial cells via measuring the conversion of [^{3}H]Arginine to [^{3}H]Citrulline. Enzymatic activity was several fold higher in the caveolar membrane fractions compared to whole plasma membrane fractions, and enzymatic activity was undetectable in non-caveolar plasma membrane. Liu et al [87] found palmitoylation was requisite for targeting of eNOS into caveolae, and from a functional standpoint stimulated $NO^{\cdot}$ production. This was assessed with an assay measuring conversion of L-arginine to L-citrulline in cell lysates treated in Ca^{2+} rich media supplemented with NADPH, BH_4, calmodulin, and L-arginine. Assay-predicted $NO^{\cdot}$ release was decreased when C15 and C26 were replaced with S residues preventing targeting to the caveolae [87]. The fact that the assay was carried out in eNOS transfected NIH 3T3 cells calls into question the applicability of this finding *in vivo* in endothelial cells. Nonetheless these findings suggest that eNOS localization into the caveolae is requisite for optimal $NO^{\cdot}$ release in response to the various stimuli that result in eNOS activation, although these findings are equivocal.

Ju et al [92] found that the N and C–termini of cytosolic Caveolin-1 bind the eNOS oxygenase domain and residues 82-101 of Caveolin-1 inhibit eNOS via allosteric inhibition of the eNOS-Calmodulin interaction. In addition aortas derived from Caveolin-1 knockout mice exhibit greater acetylcholine (ACh)-induced dilation and greater sensitivity to eNOS inhibition via L-N^G-Nitroarginine methyl ester (L-NAME) [93]. This latter finding is likely of more physiological relevance than the assays described above. However the absence of Caveolin-1 also led to hyper-proliferation of certain cell types, and in another study of a Caveolin-1 knockout, the lungs of

knockout animals exhibited uncontrolled endothelial cell proliferation and fibrosis and a resultant intolerance to exercise [94]. The ability of caveolin-1 to inhibit eNOS has also been shown to contribute to regulation of vascular permeability [95]. A recent review describes that various stimuli involved in cardiovascular disease risk (high density lipoprotein, insulin, CRP) play a role in signaling cascades that occur in the caveolae and impact eNOS activity, thus regulation of eNOS via caveolae is quite complex, but does appear to involve tonic inhibition of eNOS activity [89].

II. B. IV Guanylyl Cyclase signaling to vascular smooth muscle

$NO^{\cdot}$ primarily results in vasodilation via stimulation of Guanylyl Cyclase found in vascular smooth muscle cells [96]. Classic studies that undertook the investigation of $NO^{\cdot}$ mediated Guanylyl Cyclase activation found that $NO^{\cdot}$ activation of heme-deficient Guanylyl Cyclase was negligible, while $NO^{\cdot}$ mediated Guanylyl Cyclase activation in heme-containg forms of Guanylyl Cyclase was quite robust. [97] Gruetter et al [98] found that $NO^{\cdot}$ donors Nitroprusside and N-methyl-N'-nitro-N-nitrosoguanidine elicited dose dependent activation of Guanylyl Cyclase and relaxation in prepared bovine coronary artery strips. However, addition of other heme-proteins (hemoglobin, methemoglobin and myoglobin) abrogated the effects of $NO^{\cdot}$.

When $NO^{\cdot}$ is formed in the vascular endothelium, it rapidly diffuses into adjacent vascular smooth muscle cells and stimulates increased Guanylyl Cyclase via a conformation change induced by $NO^{\cdot}$ binding to a heme ion within Guanylyl Cyclase. Guanylyl Cyclase synthesizes cGMP from GTP by catalyzing the dephosphorylation of GTP to cGMP, thus increasing intracellular cGMP. Cyclic GMP is a second messenger that promotes vasodilation via multiple mechanisms [99]. One of these means involves K^+ ion efflux and a subsequent hyperpolarization of the vascular smooth muscle cells [100]. In rat pulmonary artery rings, cGMP-dependent dilation can be inhibited by the K_{Ca} channel inhibitor charybdotoxin or by increasing extracellular K^+

concentrations. Furthermore, in patch-clamp experiments both NO and cGMP increase whole-cell K^+ current by activating K_{Ca} channels (as shown by inhibition via charybdotoxin).

Another mechanism through which cGMP can promote vasodilation is through modulation of vascular smooth muscle cell intracellular calcium (Ca^{2+}) sensitivity. cGMP binding activates cGMP-dependent protein kinases (PKGs), which phosphorylate numerous S and T residues on many cellular signaling proteins. This can thereby result in changes in intracellular localization or activity. Many of the proteins that are covalently modified by PKG commonly regulate Ca^{2+} homeostasis and sensitivity [99].

An increase in vascular smooth muscle cell Ca^{2+} via increased flux of Ca^{2+} into the cell through membrane channels, or sarcoplasmic reticulum Ca^{2+}, results in greater Ca^{2+} binding to calmodulin. Vascular smooth muscle tone is largely determined by the activity of the myosin light chain (MLC) and cross-bridge formation between the myosin heads and the actin filaments. Myosin light chain phosphorylation at S19 is mediated by the MLC Kinase (MLCK) and MLCK is activated by Ca^{2+} bound calmodulin [101]. In contrast, MLC Phosphatase (MLCP) catalyzes the de-phosphorylation of MLC and promotes vascular smooth muscle relaxation. Thus the balance between the MLCK and MLCP is a large determinant of vascular smooth muscle tone. Cyclic GMP can increase MLCP activity [99] and PKG mediated phosphorylation can decrease activity of GPCRs (Phospholipase Cβ) that increase inositol 1,4,5-trisphosphate (IP_3) and intracellular Ca^{2+} [102]. To summarize, the vasodilatory effects of $NO^{\cdot}$ are mediated by cGMP. Cyclic GMP leads to vascular smooth muscle relaxation by activating K^+ channels which leads to hyperpolarization and relaxation of the vascular smooth muscle. Cyclic GMP also acts through PKG to decrease intracellular Ca^{2+} levels and can also activate MLCP. All of these result in downstream vasodilation.

II. C Oxidative Stress in the Peripheral Vasculature

The primary ramification of oxidative stress in measures such as FMD and in isolated resistance artery preparations is reduced $NO^{\cdot}$ bioavailability. As noted earlier, $NO^{\cdot}$ scavenging occurs when O_2^- reacts with $NO^{\cdot}$ to form $ONOO^-$ [103, 104]. In addition, O_2^- can oxidize the eNOS cofactor BH_4 to BH_2 which subsequently leads to eNOS uncoupling [105]. Uncoupled eNOS produces O_2^- in place $NO^{\cdot}$.(see figure 1B) In addition $ONOO^-$ itself has also been shown to uncouple eNOS [106]. Furthermore, $ONOO^-$ and H_2O_2 have been shown to increase arginase activity in the endothelial cells which reduces the substrate L-arginine and thus decreases $NO^{\cdot}$ production [68]. However the major source of oxidative stress in the endothelium is NADPH Oxidase. The activation of vascular NADPH Oxidase has been shown to be induced by a number of mechanisms. [67, 107] However, a well-accepted pathway involves stimulation downstream of Angiotensin II and subsequently Protein Kinase C. Therefore, the members of this pathway will be discussed at length following NADPH Oxidase.

II. C. I NADPH Oxidase

NADPH Oxidases (NOXs) are a family of multi-subunit enzyme complexes that were initially studied in phagocytes and that play a role host immune system defense against microbial and viral invasion [108-110]. phagocytic NOXs defend against pathogens via inducible (burst-like) production of ROS primarily O_2^- and H_2O_2 [108, 111]. The name of this family of enzymes is derived from the fact that NADPH is oxidized to produce O_2^- [109] The primary NOX of interest for this review is NOX II, as this isoform is thought to be the primary culprit for producing oxidative stress in the endothelium [112, 113]. Oxidase activity of the NOXs is dependent upon a

convergence of subunits, which were traditionally thought to include membrane-bound and cytosolic components.

Cytochrome b558 is a heterodimeric flavocytochrome and is the membrane-bound component of NOX II. It is collectively composed a 22 kDa α subunit commonly referred to as $p22^{phox}$ and a 91 kDa β subunit commonly referred to as $gp91^{phox}$ [114]. Apart from Cytochrome b558 phagocytic NOX II is composed of cytosolic subunits $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and the small GTPase protein Rac1/2 [110, 115]. All of the cytoplasmic subunits are multi-domain proteins except Rac [108]. Early studies on the function of phagocytic NOX II also revealed that the complex is dependent upon the substrate flavin-adenine dinucleotide (FAD). Rotrosen et al [113] used an in vitro approach to reconstitute FAD-dependent oxidase activity of the complex using only cytochrome b558, $p47^{phox}$, $p67^{phox}$, and RAC1/2. Furthermore this study found that FAD was co-localized to flavocytochrome b558, specifically $gp91^{phox}$.

Active NOX II requires assembly of the entire complex. The phosphorylation events that induce activation of NOX II involve 11 potential S phosphorylation sites in C-terminus of $p47^{phox}$ at S303-4, S310, S315, S320, S328, S345, S348, S359, S370, and S379 [116]. These S residues are convoyed with basic amino acids (Arginine and Lysine) in a region termed the polybasic region or the auto-inhibitory region (AIR; aa residues 292-340) [111]. In the auto-inhibited conformation interaction between $p47^{phox}$ and $p22^{phox}$ is prevented by tandem SH3 domain interactions that are thought to occur primarily with the polybasic region of the $p47^{phox}$ C-terminus [117]. Proline-rich regions in the C-terminus of $p22^{phox}$ (residues 151-160) are considered to be the SH3-binding motif in $p22^{phox}$ [118]. Preceding the polybasic region and the two SH3 domains is a PX domain. The PX domain primarily functions as phosphoinositide-binding domain that participates in localizing protein to membranes [119]. The C-terminus is competed by a Proline-rich motif [108, 120]. A

Proline rich or PxxP motif in the p47^{phox} C-terminus is primarily thought to serve as a binding site for the second of two p67^{phox} SH3 domains. [120] Although, in a study where all 11 of the p47^{phox} C-terminus Serines were replaced with Alanines, seven of the constructs were found to reduce agonist mediated NOX II activation [117].

Generally the GTPase protein associated with NOX II is a small 21 kDa GTPase protein (g-protein) belonging to the Rac subfamily of Rho family of GTPases known as Rac-1. In active NOX II assembly Rac1 is bound to p67^{phox} via a tetratricopeptide repeat domain in the N-terminus of the p67^{phox}. Tetratricopeptide repeat domains are 34 amino acid sequence motif, found in a wide variety of proteins and typically acts as a scaffold for inter-protein interactions, and thus facilitates in the assembly of multiprotein complexes (i.e. NOX II) [121].

Much was elucidated about the endothelial cell NOX II homolog in the decade leading up to 2000. In many of the studies that will be discussed lucigenin-elicited chemiluminescence was used to asses O_2^- production. This is an important methodological consideration as it contributed largely to the controversy regarding endothelial cell NOXII substrate preference in regard to NADPH and NADH that persisted into the early 2000s. In 1994 Mohazzab et al [112] found that O_2^- production assessed via lucigenin-elicited chemiluminescence in the bovine coronary artery endothelium was stimulated via NADPH and even more so with NADH. The Superoxide Dismutase mimetic Tiron (4,5-dihydroxy-1,3-benzenedisulfonic acid disodium salt) was used to scavenge O_2^- and lucigenin-elicited chemiluminescence was decreased. This particular experiment showed that the lucigenin-elicited chemiluminescence was specific to O_2^-. The mitochondrial complex III inhibitor antimycin and the XOR inhibitor hypoxanthine were found to have negligible effects on NADPH and NADH induced O_2^-. However Diphenyleneiodonium (DPI), a nonspecific inhibitor of flavoenzymes, was found to suppress increased O_2^-. DPI is often used to decrease

NOX enzyme-induced cellular ROS production [122] Taken together these finding s suggested that an NAD(P)H dependent enzyme and not XOR or mitochondria was the primary source of ROS in the endothelium. In 1996 Jones et al [123] showed that human umbilical vein endothelial cells (HUVECs) expressed $p22^{phox}$, $p47^{phox}$, and $p67^{phox}$ at the RNA level. Protein expression of only $p22^{phox}$ was confirmed by Northern blotting and immunoperoxidase staining confirmed expression of, $p47^{phox}$and $p67^{phox}$. Potential functionality of NOX was confirmed via the addition of NADPH to HUVEC supernatant and measurement of O_2^- via lucigenin-elicited chemiluminescence. NADPH treated supernatants produced more O_2^- and this occurrence was inhibited by the addition of DPI. A glaring limitation of the study was that the presence of cytochrome b558 could not be conclusively demonstrated. Later studies from the Shah lab further elucidated the role NOX II in the endothelium [118, 124, 125].

Bayraktutan et al [118] found that both $p22^{phox}$ and $gp91^{phox}$ are expressed in isolated rat coronary microvascular endothelial cells. At the time of the publication the controversy as to whether the enzyme complex had greater substrate affinity for NADH or NADPH persisted. This was the first paper to show that DPI specifically inhibited NADPH-induced increased O_2^- production and not NADH-induced O_2^- production suggesting that NADPH was the specific substrate for the endothelial cell NOX II homolog. Furthermore, XOR inhibitor Oxypurinol, Cyclooxygenase inhibitor Indomethacin, mitochondrial complex I inhibitor Rotenone, and eNOS inhibitor L-NMMA (L-N^G-monomethyl Arginine) had no effect on NADPH and NADH induced O_2^- production. These pharmacological experiments further reinforced the notion that NOXII was the major source of ROS in the endothelium. Limitations of the study included that the purported rat coronary microvascular endothelial cells were primary cells derived from rat hearts excised of large coronary and cardiac vessels, therefore the cells were likely more reflective of endocardium.

In 2000 Bayraktutan et al [125] published another manuscript based on molecular characterization and localization of the NOX II in the vascular endothelium once again using rat coronary microvascular endothelial cells. Both $p22^{phox}$ and $gp91^{phox}$ were cloned, cDNA was characterized and predicted amino acid structures were derived in order to compare endothelial cell NOXII with phagocytic NOX II. The subcellular location of $p22^{phox}$ and $gp91^{phox}$ was also determined.

PCR results revealed a high degree of homology between rat coronary microvascular endothelial cell $p22^{phox}$ (greater than 99% with the rat vascular smooth muscle $p22^{phox}$ and ~85% with the murine, human and porcine neutrophil $p22^{phox}$). Endothelial cell NOX II $gp91^{phox}$ was found to share >90% homology with phagocytic $gp91^{phox}$. However, several dissimilarities were found which may partially explain differences in Endothelial cell NOX II and phagocytic NOX II activity [125].

Two putative flavin-binding domains were fully conserved. However not all of the four classic four $gp91^{phox}$ NADPH-binding domains were found to be conserved in rat coronary microvascular endothelial cells. A Serine occupies amino acid residue 416 in the first NADPH-binding domain in the rat coronary microvascular endothelial cell sequence, whereas Phenylalanine in phagocyte sequences [125, 126].

Rat coronary microvascular endothelial cell $gp91^{phox}$ and phagocyte $gp91^{phox}$ sequences differed in potential glycosylation sites. Glycosylation sites are encoded by the motif N-X-(S/T), where N is Asparagine, X is any residue, S is Serine, and T is Threonine. The rat coronary microvascular cell sequence was found to contain 3 potential glycosylation sites beginning at residues N40, N97, and N430, while phagocytic $gp91^{phox}$ contains 4-5 potential glycosylation sites depending on the species of origin [125]. Another notable difference found in endothelial $gp91^{phox}$ compared to phagocytic $gp91^{phox}$ is a S substitution at residue 415 in place of Proline. In chronic

granulomatous disease a missense mutation resulting in substitution of Histidine for Proline at residue 415 is thought to contribute to the disease state by decreasing binding affinity to NADPH and significantly reducing O_2^- production [127].

Confocal immunofluorescence microscopy revealed that both endothelial cell NOX II and phagocytic NOX II displayed $p22^{phox}$ and $gp91^{phox}$ co-localization, subcellular location of the cytochrome b558 differed. In endothelial cells cytochrome b558 appeared to localize in the area of endoplasmic reticulum while previous studies found cytochrome b558 to localize in to the plasma membrane in neutrophils when induced [125] [128] Previous studies by the Shah group also revealed that unlike phagocytic cells, NOX II in endothelial cells is constitutively active producing a low level of ROS [103, 107, 118, 124, 125, 129]. Protein glycosylation normally influences enzyme activity, protein stability, and membrane trafficking, although the role of glycosylation in NADPH oxidase remains unresolved. Subcellular location of the cytochrome b558 differed in rat coronary microvascular endothelial cells relative to phagocytes. [125, 130] As noted, changes as small as single missense mutations can result in chronic granulomatous disease which results in decreased binding of NADPH to NOX II and subsequent reduction in neutrophil O_2^- production. Thus, these subtle differences in homology, glycosylation sites, and subcellular localization may explain functional differences in endothelial vs phagocytic NOX II [126].

Lastly, it should be noted that the methodology used in the Bayraktutan et al [118, 125] manuscripts did not rule out the possibility that myocardial endothelium was the primary cell type isolated rather than microvascular endothelial cells. Follow up studies performed by Li et al [124] confirmed all of the NOX II subunit are expressed at the RNA and protein level in a variety of endothelial cells from human, bovine and porcine and NADPH induced O_2^- was inhibited by DPI. Lastly it remains feasible that factors such as substrate and cofactor bioavailability[131],

differences in cytoplasmic subunit expression level of other subunits[131], and other post-translational events could also contribute to physiological differences between endothelial cell and phagocyte NOX II.

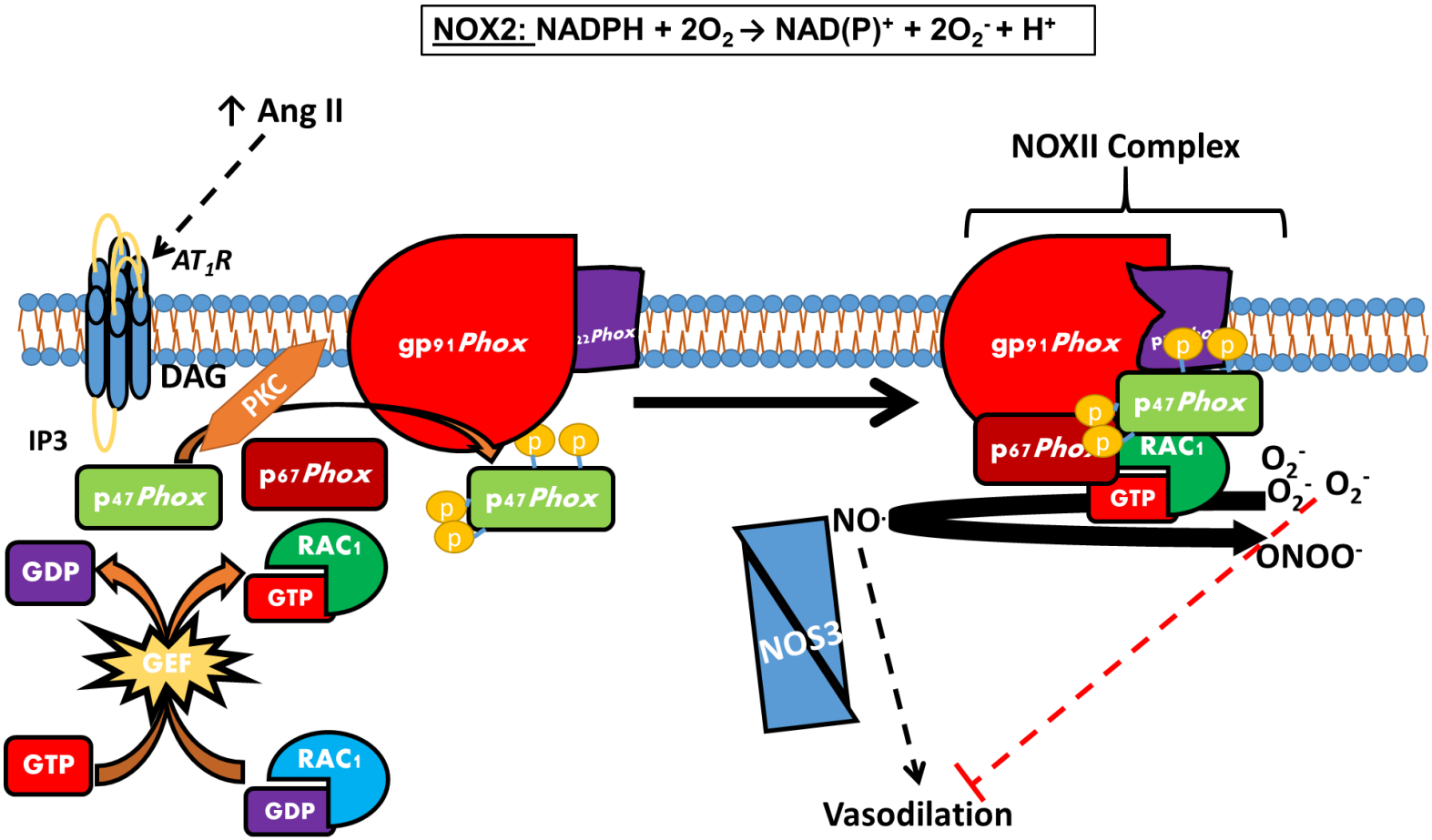

Figure 3 NADPH Oxidase II assembly in the endothelium.

Ang II binds the AT_1R, which is a GPCR. The g-protein α subunit activates phospholipase C-β (PLC-β) which hydrolyzes the phosphodiester bond between inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) in Phosphatidylinositol 4,5-bisphosphate (PIP_2). IP_3 is soluble in the cytoplasm and can diffuse to the endoplasmic reticulum, subsequently bind to a ligand-gated Ca^{2+} channel on the endoplasmic reticulum and trigger the opening of the Ca^{2+} channel. Ca^{2+} can bind to the N-terminus of Protein Kinase C (PKC-alpha, beta, zeta expressed in the endothelium) and PKC phosphorylates Serine 379 and several others Serine residues on the $p47^{phox}$ subunit of NADPH Oxidase II initiates a cascade of several protein-protein interactions modulating the recruitment of the cytosolic subunits to the Cytochrome b558 complex. Phosphorylation of $p47^{phox}$ initiates the formation of the active NOX II enzyme complex through $p47^{phox}$ docking to $p22^{phox}$. The interaction between $p47^{phox}$ and $p22^{phox}$ is normally prevented in phagocytic NOX due to an auto-inhibited $p47^{phox}$ conformation that involves dual Src homology (SH3) domains [110, 128-130]. Phosphorylation of $p47^{phox}$ relieves the inhibitory intramolecular interaction thus allowing $p47^{phox}$ to interact with the cytoplasmic tail of $p22^{phox}$ [110, 128, 129]. The ultimate outcome of active complex formation is an increase in Superoxide (O_2^-) production. The increase in O_2^- can reduce $NO^\cdot$ bioavailability by quenching $NO^\cdot$ and forming $ONOO^-$ which reduces the amount of $NO^\cdot$ to induce dilation.

II. C. II Renin-Angiotensin System

The renin–angiotensin system (RAS) plays a key role in regulating blood pressure via water and electrolyte balance, influencing the redox balance in the endothelium, and influencing neuronal and endocrine mediated vascular tone [132]. For our purposes, we were interested in Angiotensin II (Ang II) as a potent vasoconstrictor of resistance arteries at least in part through NOX II (described at length above). In regards to systemic RAS, this system begins with angiotensinogen, a 453 amino acid polypeptide secreted by the liver. Circulating angiotensinogen is enzymatically cleaved to the 10 amino acid peptide angiotensin I (ANG I) by the kidney-derived enzyme renin. ANG I is then cleaved to the eight amino acid effector hormone angiotensin II (ANG II) by angiotensin converting enzyme (ACE) [133]. Prior to the 1940s, renin was thought to directly cause high blood pressure before the discovery of angiotensinogen, ANG I and ANG II [134]. Dating as far back as the 1870s an association between nephritis and high blood pressure (measured using sphygmographs) was made [134]. In studies performed at the end of the 1800s, Tigerstedt and Bergman [134] demonstrated a pressor compound in the renal tissue of the rabbits and coined the name renin based off the compound's renal origins. Renin could be extracted with glycerin and was destroyed by boiling and was found to induce a prolonged pressor response. In a seminal paper published in 1935 Goldblatt et al [135] elucidated that experimentally induced mild renal artery ischemia was sufficient to induce elevated systolic blood pressure systemically without concomitant decrements in renal function. A follow up study using experimentally induced mild ischemia localized to the kidneys found that complete sympathectomy (preceding the renal artery clamping) did not reduce the severity of hypertension. This finding indicates that this model of hypertension was based on a circulating factor. Harrison et al [136] then found that kidney extracts

from dogs with the experimental model of hypertension increased blood pressure when injected into rats, while the kidney extracts from control dogs did not. In the following years a compound in the renal vein blood of ischemic kidneys was also found to induce hypertension when injected into naive animals. However this unidentified compound was extracted from blood with 70% acetone and had a short pressor effect, which was in contrast to renin (glycerin extraction; prolonged pressor effect). The final conclusion was that renin acted enzymatically on a plasma protein to produce the unidentified compound, which would later be dually coined angiotinin and hypertension, and eventually angiotensin [134].

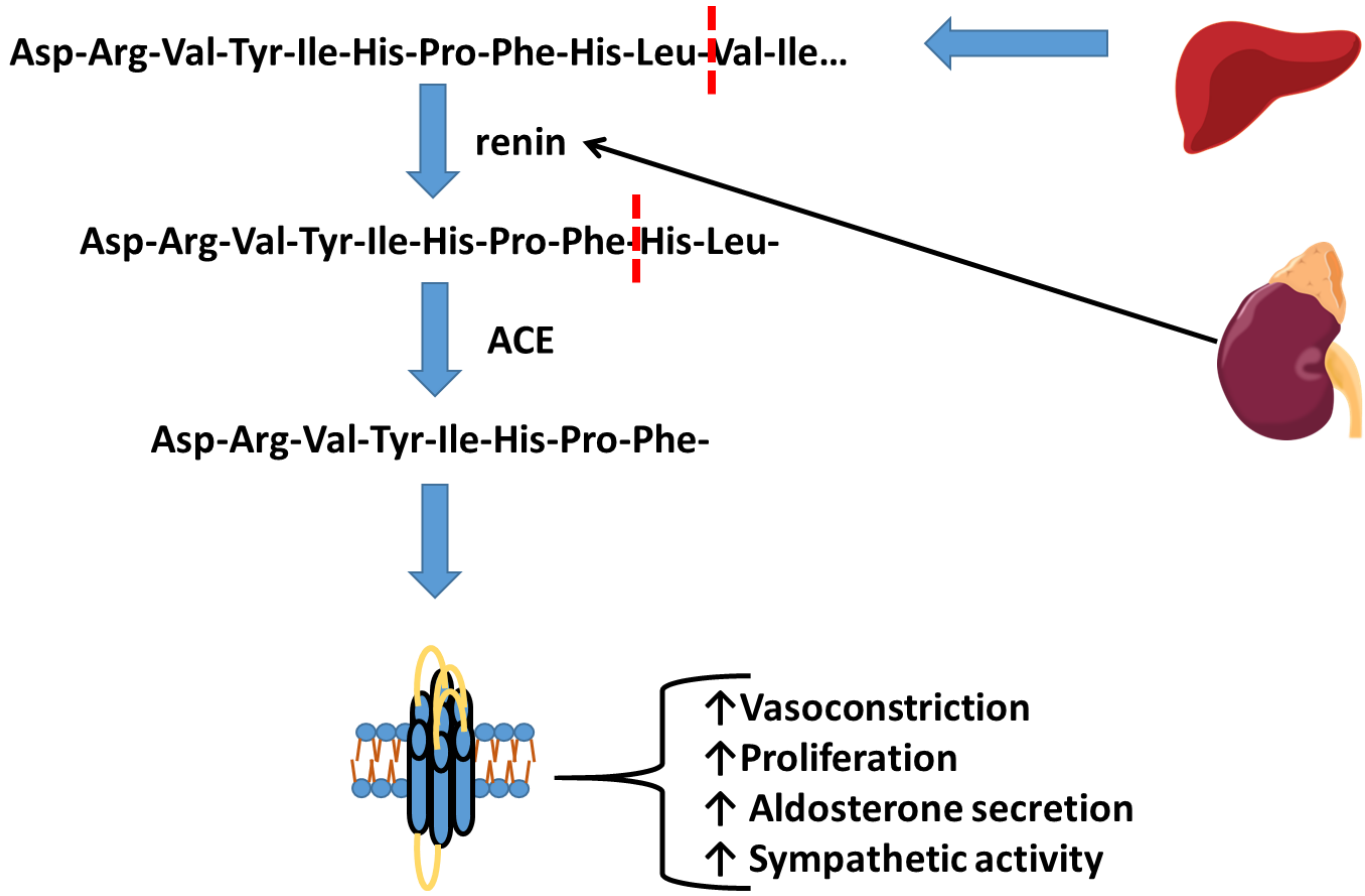

Figure 4 Simplified Depiction of the Classical Renin Angiotensin System.

The protein angiotensinogen released by the liver is converted to Angiotensin I via the plasma protein renin. Renin is synthesized from juxtaglomerular cells in the kidneys. Angiotensin I is subsequently converted to angiotensin II by the enzyme angiotensin-converting enzyme (ACE) found primarily in the capillaries of the lungs. Angiotensin II is an octapepetide with potent vasoconstrictor properties when it binds to the Angiotensin type 1 receptor.

The renin-angiotensin system has historically been viewed to act in an endocrine fashion. However the importance of a local RAS is also well described. As far back as 1976 Caldwell et al [137] used an antibody to ACE and demonstrated that it was expressed in the endothelium of rabbit lung, liver, adrenal cortex, pancreas, kidney, and spleen, while several parenchymal cells from these organs were found not to express ACE. Subsequent studies investigating aortic tissue from several species subjected to collagenase digestion, in order to separate the endothelium from

vascular smooth muscle and adventitia, to study ACE activity within each vascular tunic ACE was found to be preferentially expressed by the endothelium. An N-Hippuryl-His-Leu assay was used to detect cleaved Histidine-Leucine sequences, the other product of ACE action apart Ang II. (see figure 5). [138] In contrast, a future studies found the adventitia of several arties including the aorta to express ACE [139]. Furthermore, ACE activity has been found in denuded vessels suggesting that while the highest amount of expression may be in the endothelium, ACE localization is not exclusive to the endothelium in blood vessels [140, 141]. The presence of an endothelium specific RAS was supported in 1989 by Mizuno et al [142] when physiologically relevant levels of ANG I and ANG II (41.9 ± 7.4pg and 63.4 ± 12.0pg, respectively) were detected in the perfusate of HUVECs using high-performance liquid chromatography. In the same study when the ACE inhibitor captopril was added to the Krebs-Ringer solution production of Ang II was significantly blunted, although not completely attenuated, suggesting anther means of converting Angiotensin I to Ang II. Several studies have since elucidated the involvement of not only ACE but a chymostatin-sensitive angiotensin II-generating enzyme at the tissue level [143-145].

In regards to renin and angiotensin in the local RAS, the findings of ANG I and ANG II discussed above suggests that local production of these two components does occur at the tissue level. In addition the localization of angiotensinogen has been shown in the vascular smooth muscle and perivascular adipose, at least at the mRNA level [146]. Perfusion of isolated, bloodless, ringer solution-perfused rat hind limb preparations with renin has been shown to cause a dose-dependent Ang I and Ang II release and a subsequent vasoconstriction, suggesting the presence of angiotensinogen. Inhibition of renin activity abolished the increase in ANG I and ANG II [147].

Extrarenal detection of renin has also been determined albeit using RNA [148, 149]. Renin expression (measured by immunofluorescence) and renin like activity has also been shown in cultured vascular smooth muscle cells [150]. Studies using isolated hind limb preparations to demonstrate that infusions of synthetic tetradecapeptide renin substrate can induce increases in perfusion pressure similar to infusions of ANG I or ANG II, suggest both ACE and renin activity in the vasculature [145]. While there has been some pushback regarding the validity of the use of the synthetic tetradecapeptide renin substrate because several enzymes (including isorenin, pseudorenin and cathepsin D) have been shown to cleave this substrate, the optimal pH at which these alternative enzymes operate is not close to physiological pH (of course the operating point of renin is) [151]. Furthermore Kilgers et al [152] demonstrated the release of angiotensinogen and the formation of ANG I and ANG II in a bloodless, perfused, isolated rat hind limb preparation when renin was added to the ringers solution. The release of angiotensinogen was detected in the perfusate by radioimmunoassay and further confirmed by Western blot. However exogenous angiotensinogen infusion increased the production of ANG I and ANG II several fold and bilateral nephrectomy 24 hours preceding the experiments reduced basal angiotensin release below the detection limits. Other studies have also shown that following nephrectomy the antihypertensive effects of renin inhibitors lose their effect [153]. Taken together these findings suggest that the vascular wall does contain renin and angiotensinogen but the vast majority of renin and angiotensinogen are taken up from the circulation [152-154]. Studies have shown that renin and angiotensin can enter HUVECs through diffusion [155, 156]. Thus high blood pressure may facilitate local RAS by facilitating diffusion of renin and angiotensin [36, 41, 157].

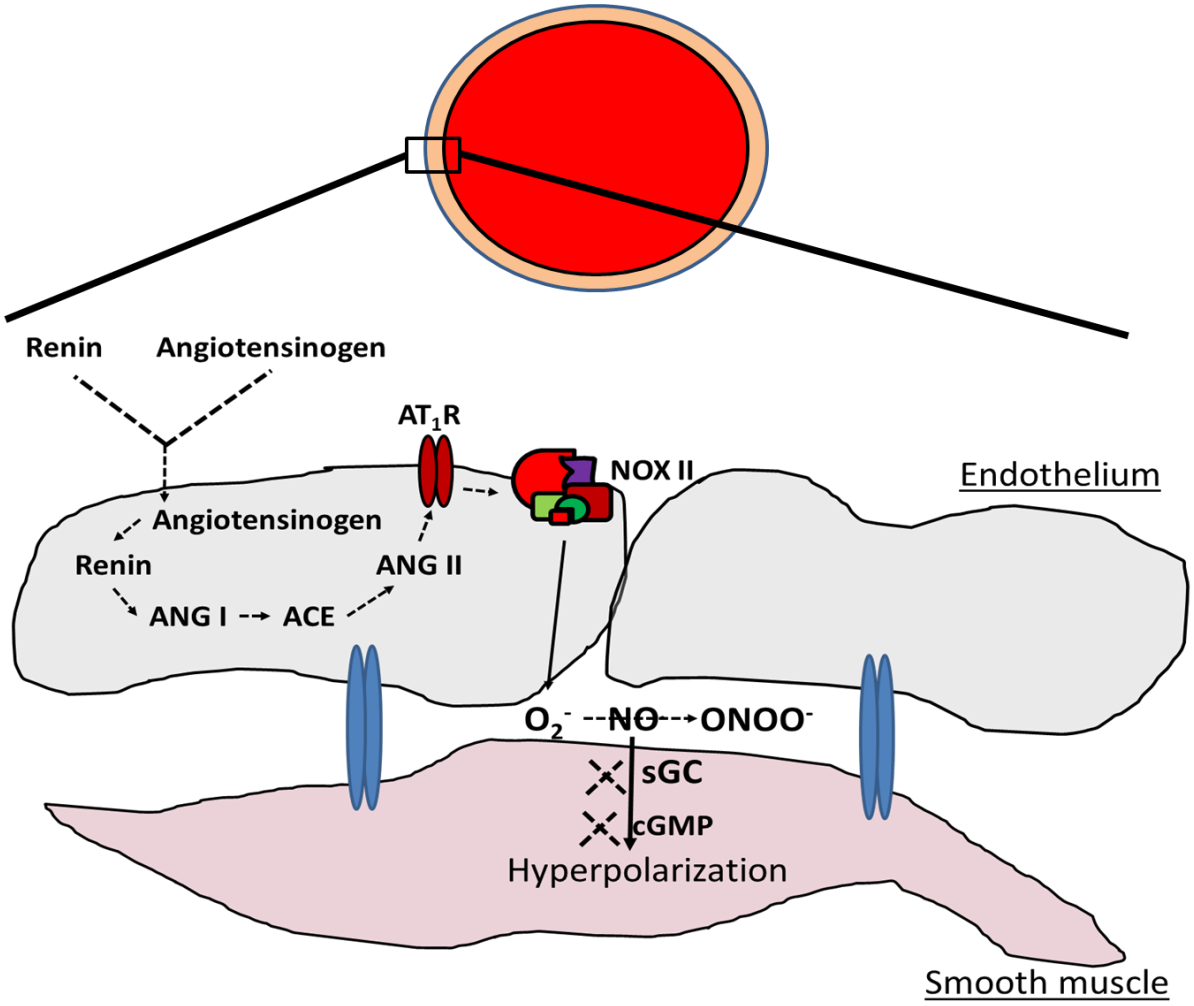

Figure 5 A proposed model of the local Renin Angiotensin System.

Isolated vessels have been shown to express Angiotensin Converting Enzyme (ACE). ACE cleaves the primary effector Ang II. Ang II promotes oxidative stress primarily through NADPH Oxidase II resulting in decreased $NO^{\cdot}$. The Ang I substrate for ACE is provided by cleavage from Angiotensinogen via Renin. Both Angiotensinogen and renin are thought to diffuse from the blood into the endothelium and interstitial space within the vessel.

II. C. III Protein Kinase C

The link between RAS, ANG II, and the activation of NOX II in the endothelium is Protein Kinase C (PKC). The PKC family is a ubiquitous family of Serine and Threonine protein kinases that play a role in a large number of cellular signaling pathways. Signals that stimulate members of the large families of GPCRs (i.e. AT_1R), receptor tyrosine kinases, and non-receptor tyrosine kinases have all been shown to activate PKC [158-161].

Most cells express more than one isoform of PKC and the various PKC isoforms mediate different cellular events (described below and in figure 6) [162]. The various PKC isoforms all consist of N-terminus regulatory domains and C-terminus catalytic domains. All PKC isoforms also contain of a pseudosubstrate region which is a small amino acid sequence lacking Serine and Threonine. This pseudosubstrate region binds the substrate binding pocket in the catalytic domain, thus keeping the enzyme inactive. When the regulatory N-terminus domains are recruited to the plasma membrane auto-inhibition is relieved as the pseudosubstrate region no longer occupies substrate binding pocket in the C-terminus. Thus the catalytic domain of PKC is free to exert its action on target substrates, such as $p47^{phox}$ [163, 164]. The regulatory N terminus contains two pairs of zinc fingers within which serve as the binding site for DAG and phorbol esters. In fact many of the papers used to characterize endothelial NOX II (described above) used Phorbol 12-myristate 13-acetate (PMA) and similar esters to stimulate NOX II via the PKC pathway [111, 165, 166]. Cleaved IP_3 enters the cytoplasm and activates IP_3 receptors on the endoplasmic reticulum, which opens $Ca2^+$ channels on the endoplasmic reticulum, allowing mobilization of $Ca2^+$ into the cytosol. In the absence of cofactors, such as DAG and IP_3, PKCs are generally maintained in their auto inhibitory state via binding of the pseudosubstrate region to the substrate binding pocket in the C-terminus.

The PKC family members can be divided into subtypes based their regulatory domains and cofactor requirement [160]. Differences in regulatory domains and cofactor requirements result in different substrate specificities. Deciphering the specific functions of isozymes has not been determined yet and will likely not be until specific inhibitors of each isozyme are developed. [167] These subtypes include the classical PKCs (cPKC), novel PKCs (nPKC), and atypical PKCs (aPKC).

Classical PKCs include alpha (α), beta (βI & βII), and gamma (γ) isoforms and require Ca^{2+}, phosphatidylserine and DAG or phorbol esters for activation. Novel PKCs include the delta (δ) epsilon (ε), eta (η), and theta (θ) isoforms and differ from cPKCs in that they do not require Ca^{2+} for activation. The regulatory region of cPKCs and nPKCs contain the C1 domain which contains two Cysteine rich motifs and serves an important role in forming an ester bond with DAG binding or Phorbol ester binding. Both cPKCs and nPKCs contain a C2 domains as well but The C2 domain of nPKC lacks amino acids involved in Ca^{2+} binding thus some in the PKC field refer to this as the "C2-like domain" [161].

Atypical PKCs include the iota (ι), lambda (λ) and zeta (δ) isoforms and are insensitive to Ca^{2+}, phosphatidylserine or DAG [70, 164]. Atypical PKCs lack the C2 domain. In addition they only contain only one Cysteine-rich motif, rather than two ,such as cPKCs and nPKCs [161]. However aPKCs contain a Phox and Bem1 (PB1) domain unlike cPKCs and nPKCs. The PB1 domain plays a role in mediating heterodimerization in NADPH Oxidase cystoplasmic subunits but its role in aPKC signaling with $p47^{phox}$ does not appear to have been elucidated [168].

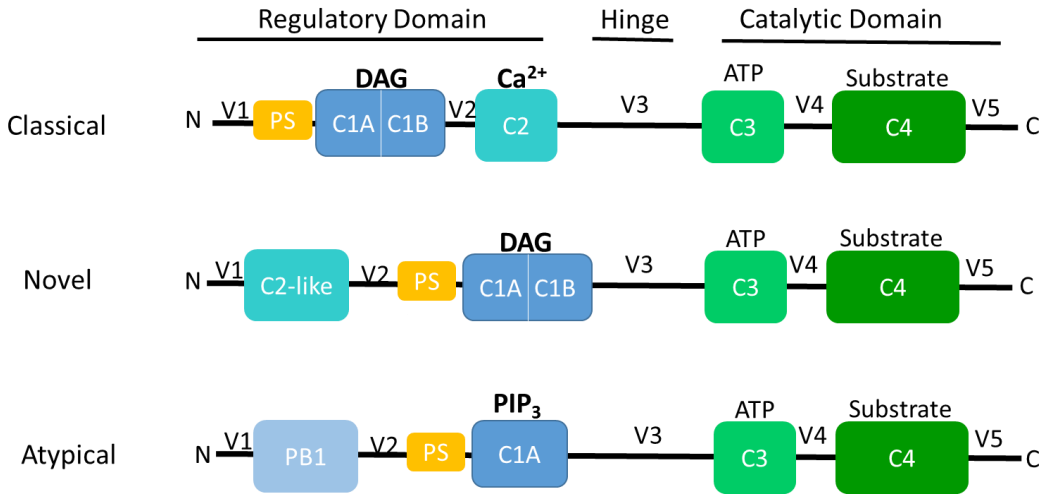

Figure 6 Character of Protein Kinase C Isozymes.

Members of the PKC family are composed of a single polypeptide chain, comprised of an N-terminus regulatory region (~20-40 kDa) and a C-terminus catalytic region (~45 kDa) The PKCs contain four conserved domains: C1-C4. C1 domains are known as DAG/phorbol ester binding domains. They consist of two cysteine rich motifs which contribute to two zinc fingers in the regulatory domain. Each of the two zinc fingers is composed of six cysteine residues and two zinc atoms. A C2 domain is structural domain involved in targeting proteins to cell membranes. The typical C2 (as characterized in PKC) is composed of 8 β-strands that coordinates with two or three Ca^{2+} ions following IP3- induced elevations in cytosolic Ca^{2+}. C3 is the ATP-binding lobe and C4 is the substrate-binding lobe of the catalytic region. Illustration and information inspired by references [161, 162, 164, 169]

In regards to activation of PKC by Ang II, Ang II binds the Angiotensin I receptor (AT_1R), a GPCR which activates phospholipase C. Phospholipase C(β) catalyzes the hydrolysis of PIP_2 to IP_3 and DAG which act as secondary messengers [84, 159]. PIP_2 is a phospholipid component of cell membranes and also serves as a substrate for many cell signaling proteins [170]. In regards to the specific isoforms thought to play a role in $p47^{phox}$ phosphorylation, PKC α and β (classic), δ (novel), and δ (atypical) all have all been shown to do so [165] and in another study endothelial cells were shown to express PKC α and β, and δ the at the mRNA level and protein level [171].

II. D Regular Exercise and vascular adaptations

The scientific literature suggests that only ~60% percent of the risk reduction in CVD associated with exercise can be accounted for by a reduction in traditional risk factors [19, 59]. The remaining ~40% is likely at least in part due to "vascular conditioning", whereby a combination of mechanical stimuli, in addition to changes in circulating factors, ultimately render the vascular more robust against disease [172]. The mechanisms underlying vascular conditioning involve an up regulation in anti-oxidative enzyme content and a decrease in pro-oxidative enzyme expression and some studies have shown exercise to confer this protective effects on the actual vascular wall [173, 174]. Shear stress independently seems to play a major role in vascular conditioning.

The shear stress profiles the endothelium is exposed to will vary according to metabolic demand (i.e. sedentary vs exercise). Shear stress modulates endothelial function via mechanotransduction, which describes the transduction of biomechanical forces into cellular signaling cascades within the endothelium via the cytoskeleton. There are various mechanosensors on the endotelial cell membrane including the glycocalyx, ion channels, and GPCRS among others [175, 176]. The phenomenom of rapid onset vasodilation with exercise is an *in vivo* measure demonstrating the sensitivity of the endothelium to respond to a rapid increase in shear stress [177]. Furthermore, inhibition of potassium inward rectifier (Kir) channels has been shown to supress rapid onset vasodilation to a muscle contraction illustrating the importance of ion channels [178]. However, single thigh cuff compressions used to mimic the mechanical component of a single contraction produces blunted hyperemia compared to single muscle contractions, indicating metabolic factors also likely play a role in rapid onset vasodilation [177]. However, using an *in vitro* model of shear stress in cells does allow for reductionist studies into the effect of shear stress

albeit this method inherently ignores the importance of the heterocellular interaction between the endothelium and vascular smooth muscle cells. [179]

II. D. I Shear stress effects in endothelial cells

Several studies have shown that subjecting cultured endothelial cells to high physiologic shear stress ($\geq$ 20 dynes/cm^2) induces beneficial adaptations, such as adopting a more anti-atherogenic phenotype along with concomitant increase in expression of anti-oxidant enzymes and decrease in expression of pro-oxidant enzymes. In a study by Uzarski et al [180] HUVECs exposed to pulsatile bursts of shear stress modeled to mimic from real-time variations in heart rate. These HUVECs were compared to HUVECs subjected to steady state laminar shear stress. In both conditions eNOS mRNA expression was significantly increased after 24 hours. Perhaps the most telling experiment in this study was a functional assay used to determine adhesiveness for leukocytes (GFP+ HL-60 cells) after four hours of endothelial cell activation with TNFα (immediately post 24 hour shear or static culture) and shear exposed HUVECs demonstrated reduced HL-60 leukocyte attachment compared to cells grown in the static condition. Boo et al [82] found that shear stress increases eNOS phosphorylation at S1179 and S635. Inhibition of the PI3 Kinase/Akt pathway with wortmannin did not affect shear stress mediated phosphorylation of eNOS at S635 while inhibition of PKA with H89 or knocking down PKA expression with a recombinant adenovirus did. However wortmannin inhibition did reduce shear induced phosphorylation at S635. The exact role of PKA mediated changes in response to exercise has not been fully elucidated but it may play a role in more efficient calmodulin binding and electron transfer [82, 181]. Taken together these results suggest shear stress may enhance eNOS expression

and reduce susceptibility to atherogenesis. Further studies have demonstrated the ability of shear to promote endogenous anti-oxidant capacity.

A number of studies have demonstrated high shear stress increased expression of the Superoxide Dismutase (SOD) in endothelial cells. SOD enzymes catalyze the dismutation of O_2^- into H_2O_2 thus increasing $NO^{\cdot}$ bioavailability. SOD I is located in the cytoplasm, SOD II is found in the mitochondria, and SOD III is extracellular. SOD I and SOD III complex with copper and zinc, whereas SOD II complexes with manganese [182]. In regards to shear stress in cultured cells regulating SOD, HUVECs subjected to 15 dynes/cm^2 were found to express increased SOD I at the RNA and protein expression in addition to increased NOS levels [183]. Feairheller et al [23] found that 24 hours of exposure to 20 dynes/cm^2 increased SOD II and total SOD ptrotein content in HUVECs derived from African Americans although not in HUVECs derived from caucasians. Exercise may also confer beneficial adaptation to the mitochondria which appear to play a critical role in endothelium function [184, 185].

Kim et al [186] used a 20 dynes/cm^2 shear stress intervention to increased expression of genes related to mitochondrial biogenesis including NRF-2 and the mitochondrial complexes involved oxidative phosphorylation coupling in HUVECs and human aortic endothelial cells. Furthermore, the mitochondria membrane potential was reduced by 72 hours of shear stress. This is of importance as mitochondrial membrane hyperpolarization is associated with excessive oxidative stess and impaired endothelium function [184, 187, 188]. In a translational study by Kim et al [189] shear stess was found to mimic the effects of an exercise intervention in prehypertensive participants. Not only did shear induce mitochondrial biogenesis markers, but levels of endothelial cell microparticles (e.g. CD31$^+$) were reduced and these effects were abolished using small interfering RNA transfection to knockdown Sirtuin 1. This finding is interesting in that it may shed

light on the potential insulin sensitizing effect of shear stress [190]. Shear stress is also associated with a decrease in proteins involved in oxidative stress and vascular dysfunction.

Studies have also show that shear reduces NOX II subunit expression [23]. In particular 20 dynes/cm^2 shear stress has been found to reduce p47^{phox} subunit expression in HUVECs derived from African Americans. Twenty four hours of 30 dynes/cm^2 in HUVEC decreased O_2^- production, as measured via a cytochrome c assay, and reduced p47^{phox} and gp91^{phox} expression at the mRNA and protein level. eNOS expression was also increased and NO$\cdot$ prodction (Griess reaction performed using HUVEC perfusate) was also increased. Furthermore inhibition of eNOS derived NO$\cdot$ production by L-NAME prevented downregulation of p47^{phox} and gp91^{phox} . Interestingly this study found no effect of shear on SOD I expression. Taken together these findings suggest NO$\cdot$ levels paradoxically inversely regulate NOX II expression. However these findings do not appear to have been replicated at this time [191].

Endothelin-1 (ET-1) is a peptide that acts through GPCRs as a potent vasoconstrictor and promoter of vascular smooth muscle cell proliferation [192]. Endothelial cells convert preproendothelin to proendothelin, which is the precursor to mature ET-1 via endothelin converting enzyme I (ECE1). One study investigating the effects of 24 hours high shear exposure (30 dynes/cm^2) in HUVECs found reduced preproendothelin and ECE1 mRNA levels [193]. Another investigation on the effects of 24 hours of shear stress (20 dynes/cm^2) also found preproendothelin expression was reduced HUVECs [194]. More importantly both studies found a reduction in ET-1 release as measured in the HUVEC perfusate. While providing insight into shear stress modulation of the endothelium, the cells in the experiments described above, are being investigated outside of their normal context in the vascular wall. In addition this technique negates

non shear mdiated components of exercise. Therefore the studies described below involve the use of intact blood vessels.

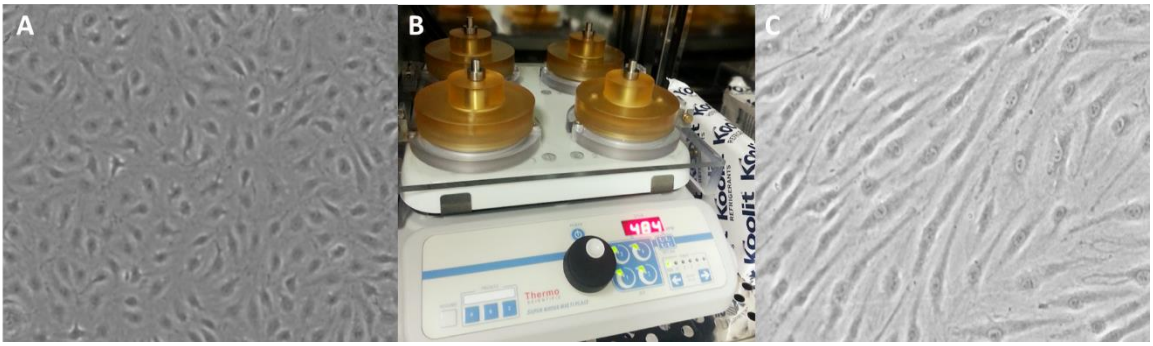

Figure 7 The effects of laminar shear stress on endothelial cells.

(A) Human adipose microvascular endothelial cells (HAMECS) raised under static conditions exhibit a "cobblestone" morphology. **(B)** An image of the cone and plate apparatus developed by Dr. Michael Brown's Vascular Health Laboratory used to subject endothelial cells to shear stress. **(C)** HAMECs adopted an elongated fusiform morphology in the direction of flow after being exposed to 24 hours of 20 dynes/cm^2 shear stress.

II. D. II The effects of exercise on isolated arteries

In a study undertaken to determine the role of acute exercise on vascular signaling kinases and their subsequent role on eNOS phosphorylation mice were subjected to 50 minutes of treadmill running. Subsequently isolated vessels were used to measure the phosphorylation of Akt, AMP Kinase (AMPK), and cyclic adenosine monophosphate response element binding protein (CREB) – a downstream mediator of protein kinase A (PKA) activation. In addition eNOS phosphorylation at S residues 617 (specific to Akt), 635 (specific to PKA), and 1177 (Akt, PKA, and AMPK) were measured along with Threonine (T) 495 in the presence or absence of wortmannin, a pharmacological inhibitor of PI3K/Akt. eNOS S1177 phosphorylation was increased from microvessels obtained post-treadmill running animals relative to microvessels

obtained for sedentary counterpart mice. Acute exercise was also associated with increased phosphorylation of Akt (S473), CREB (S133), AMPK (T172), and eNOS at S617, but not at S635 or T495. The lack of S635 can be interpreted as lack of PKA pathway activity [80]. However in the presence of wortmannin (via drinking water) Akt phosphorylation and eNOS phosphorylation at S617 during acute treadmill running were reduced. Increased AMPK and CREB phosphorylation were unchanged relative to mice who underwent acute exercise without wortmannin treatment. Microvessel eNOS phosphorylation at S1177 remained elevated following exercise after wortmannin treatment relative to values obtained from sedentary animals, but was decreased by roughly 50% compared to exercised mice how were not given Wortmannin. These data suggest that the PKA pathway may not play a major role in acute exercise mediated activation of eNOS. However specific inhibition of the PKA was not undertaken therefore this supposition is not entirely supported. In addition others have found shear stress mediated activation of eNOS to rely predominately on PKA [81, 181]. Nonetheless this study provides insight into the mechanisms through which exercise may improve $NO^{\cdot}$ signaling. Apart from improved $NO^{\cdot}$ signaling exercise has been shown to improve the redox environment in arteries from animals subjected to regular exercise.

Rush et al [173] found that a four month exercise program in pigs increased SOD I protein expression in primary endothelial cells isolated from the aorta and increased SOD I activity in homogenates derived from the whole aorta compared with primary cells and aortic homogenate derived from sedentary pigs. NOX II subunit $p67^{phox}$ expression was also reduced. In a separate study by Rush et al [195] using the same intervention in pigs, SOD I at the RNA and protein l expression was increased in isolated coronary arteriole homogenate derived from exercised pigs compared to control pigs. The vessels from exercised pigs also exhibited greater SOD activity

measured in tissue homogenate. However there was not an effect of exercise on SOD II or p67^{phox} expression.

Durrant et al [113] found that ~12 weeks of voluntary wheel running in mice improved endothelium dependent dilation to Acetycholine (ACh) in carotid arteries in an NO$^{\cdot}$ dependent fashion, as indicated by blockade with L-NAME. Dilation to ACh and sensitivity to NO$^{\cdot}$ were improved in cage control (relatively sedentary) mice via pre-treatment with the SOD mimetic Tempol and the antioxidant Apocynin suggesting that improving SOD activity or decreasing NOX II activity are beneficial for endothelium function. The wheel running mice also exhibited increased SOD III protein expression, but wheel running did not influence SOD I or SOD II expression. SOD II activity and total SOD activity was increased in a subset of older mice. Wheel running reduced p67^{phox} expression in a subset of older mice but not younger mice. A large baseline differences in p67^{phox} in young vs older mice suggest this likely had to do with the law of initial values. However wheel running did decrease presumed NOX II activity as less ROS was detected in artery lysates using an Amplex Red assay. Exercise also appears to imporve mitochondrial health in the vasculature as well.

Similar to the shear studies in cells mentioned above, Park et al [196] found five weeks of swimming to improve markers of mitochondrial biogenesis and mitochondrial content in arteries from the swimming mice compared to controls. SOD II protein expression was increased and ROS production as measured via dichlorofluorescein diacetate fluorescence was decreased. ACh induced dilation was not impacted by the swimming however the vessels from these mice did express greater phosphorylation eNOS s1177 and AMPK T172 and less sensitivity to phenylephrine induced vasoconstriction. Interestingly Akt S473 phosphorylation was not affected suggesting most of the improvement in NOS signaling was via the AMPK pathway. The results

that shear stress in cultured endothelial cells can elicit similar adaptations as those found in whole body exercise studies suggests that shear stress and the resultant mechanotransduction of this stimulus into the vasculature are key mediators of exercise related adaptations in the endothelium. Interestingly, evidence from humans seems to support this as well.

II. D. III Shear stress modulation in human studies

In order to investigate the effects of regular exposure to increased shear stress on endothelium function Naylor et al [197] recruited nine healthy men to undergo a forearm heating protocol in warm water (42°C) three times a week for eight weeks. A pressure cuff was inflated around one of each of the participants' forearms (100mHg) to suppress the heat-induced increase in shear stress. The forearm heating intervention elicited an increase in brachial artery FMD, with significant differences witnessed as early as week two of the intervention. Furthermore, the forearm heating intervention improved FMD to ischemic handgrip exercise. None of these positive adaptation occurred in the arms where the forearm was cuffed to impede an increase in shear stress. Nitroglycerin mediated dilation was not affected by the forearm intervention suggesting these adaptations were confined to the endothelium. Using an identical protocol as described above, Green et al [198] found that the eight week forearm heating intervention improved forearm cutaneous microvasculature's ability to dilate to heat, as measured by laser doppler flowmetry, in the uncuffed arms but not the cuffed arm.

Using similar methodology to Naylor and Green, Tinken [22] used a pressure to cuff to suppress exercise induced increases in shear to investigate if this would prevent endothelium responses/adaptations induced via exercise. Bilateral brachial FMD was measured in healthy young men before and after 30-minute interventions of recumbent leg cycling and bilateral handgrip exercise, and to bilateral forearm heating. During each intervention, a cuff was inflated

(60 mmHg) around one forearm to suppress the concomitant increase in shear stress expected by each intervention. In the uncuffed arm shear stress was increased by all interventions and to a similar magnitude. All three of the interventions improved brachial artery FMD. Neither an increase in shear stress nor an improvement in FMD was exhibited in the cuffed arm. In a follow up exercise intervention study ten healthy men underwent eight weeks of four times a week of progressive bilateral hand gripping exercise [21]. A cuff was placed around one of the forearms during all training sessions to prevent exercise induced increases in shear stress. Similar increases were observed in grip strength and forearm muscle cross sectional area in both limbs. However, training only increased brachial artery FMD in the uncuffed limb with significant differences occurring as early as two weeks into training. Furthermore, recent studies suggest that a reduction in popliteal artery FMD that occurs with prolonged sitting is prevented by lower limb heating [199] and fidgeting [200], both of which increase shear stress through the popliteal artery.

The studies highlighted in this section suggest that exercise improves $NO^{\cdot}$ bioavailability and promote a favor redox environment that promotes vascular homeostasis. Shear stress in cultured cells elicits many of the same adaptations as whole body exercise does in isolated vessels and manipulation of shear seems to influence clinical measures of endothelium function. Taken together these findings suggest that an increase in blood flow to active tissues during exercise wisa major mediator of vascular conditioning. However, it should be noted that exercise has been shown to alter flow patterns in a systemic fashion [201] and adaptations are made beyond arteries in active skeletal muscle with training [172].

II. E Obesity and the vasculature

Obesity is a major public health burden in the United States. More than two-thirds of adults are overweight or obese, with over half of these individuals being classified as obese (BMI ≥ 30 kg/m^2) [202]. Obesity increases the risk for chronic diseases such as hypertension, Type II diabetes, and CVD [203]. Obesity is also linked to increased mortality from all causes [204]. A central distribution of adipose in the trunk is specifically deleterious in predisposing individuals to chronic disease [205-207]. Part of the pathogenesis is an accumulation of excess lipid metabolites in cells and organs, particularly diacylglycerol and ceramide, resulting in inflammation and oxidative stress [203]. Increased DAG levels can activate PKC, which phosphorylates eNOS T495, and negatively regulate eNOS [83]. Therefore the accumulation of fat itself may not be damaging but anatomic and functional abnormalities within adipocytes that occur during obesity may contribute to the development of disease [208].

In regards to inflammation, adipose tissues secretes adipose-specific cytokines are referred to as adipokines [209]. These adipokines play a large role in influencing inflammation [17]. Obesity is associated with a more pro-inflammatory secretion profile of adipokines, although a large degree of heterogeneity exists [210]. In addition increased accumulation macrophages within the adipose tissue during obesity may play role in the secretion of pro-inflammatory cytokines [211]. This pro-inflammatory phenotype is thought to promote CVD through perivascular modulation of blood vessel and myocardial function [212, 213]. Histological studies have shown obesity in murine models is associated with greater perivascular adipose tissue in close proximity to the walls of blood vessels [214] and supernatant from these perivascular adipocytes has been shown to produce adipokines that promote vascular smooth

muscle cell proliferation [215]. Furthermore excessive adipose tissue and an inflamed adipose phenotype have been shown to be linked to impaired brachial artery FMD compared to subjects who did not demonstrate an inflamed adipose phenotype [210].

Obesity and the resultant increase in lipid metabolites are thought to contribute to oxidative stress in part through increased expression of specific isotypes of PKC and NADPH Oxidase. (PKC and NOX II discussed at length above) [208] Activation of vascular PKC is associated with endothelium dysfunction and insulin resistance in obese murine models [216]. Perivascular adipose surrounding murine mesenteric arteries has been found to express NOX II subunits. Removal of this perivascular adipose suppresses vasoconstriction to agonists, as does inhibition of NADPH Oxidase [217].

Recent evidence suggests that an increase in leisure time physical activity and exercise are two primary factors that could be used to address the obesity epidemic [218]. This is logical as energy balance is result of caloric intake and expenditure. Weight loss generally necessitates a reduction of dietary caloric intake and an increase in physical activity [219]. Furthermore, studies indicate that sustained engagement in regular exercise training promotes maintenance of weight loss [220, 221]. Current recommendations suggest that health benefits can be obtained from a minimum of 150 minutes of moderate intensity aerobic exercise per week [220]. However obese individuals may benefit from engaging in resistance exercise during their weight loss regiment in order to retain lean muscle mass and further facilitate an improvement in body composition [220]. Indeed our group has conducted such a study [27]. Nonetheless increases in aerobic fitness supersede weight loss in regards to CVD risk reduction [206]. This may be in part due to the ability of exercise to preferentially decrease ectopic fat thereby reducing infammation

[222]. Exercise results in greater systemic blood flow and liberation of FFAs from the adipose tissue and this is thought to improve the accumulation of metabolic intermediates and the metabolic state of adipose tissue [223].

II. F Post-acute Exercise vascular responses

The beneficial health effects of regular exercise are indisputable [9]. However, the effects of acute bouts of exercise on the vasculature are not as well studied. Observational studies suggest acute strenuous physical exertion increases the risk of cardiovascular events in sedentary adults [24, 25]. The experimental evidence on this matter is equivocal [26]. Some studies show that acute exercise impairs vascular function [27-30, 34, 36-38, 209, 224-231], some studies show no effect [30, 34, 36, 37, 226, 229, 231-233], and some studies show an increase in vascular function following acute exercise [22, 37, 209, 230, 233-236]. The divergence in findings is due to a number of factors including differences in participant sex, age and health/fitness status, varied intensities of the acute exercise stimulus, and assessments being taken at varied time pointsalso contribute to the divergent finds in the literature. These studies will be concisely summarized here (see table 2.1), followed by mechanistic considerations.

Training status is an important consideration in regards to the post-acute exercise vascular response. Harris et al [38] found a decrease in brachial artery FMD one hour following exercise independent of intensity (25%, 50%, or 75% VO_{2Max}) in unfit overweight male participants. However fit overweight male participants experienced an increase in brachial artery FMD. Our group found similar findings in overweight and obese females. Prior to eight weeks of circuit resistance training overweight and obese females demonstrated a decrease in brachial artery FMD following acute leg press exercise. However eight weeks of training improved fitness (as indicated

via VO_{2Max}) and prevented a reduction in brachial artery FMD following acute leg press exercise [27]. Hwang et al [30] also found that regular exercisers were protected against an acute exercise induced decrement in vascular function while sedentary subjects were not. Four cross sectional studies from our group have also found that sedentary subjects are vulnerable to a decrease in post-acute resistance exercise decrease in vascular function while trained individuals experience no decrement or an increase [34, 36, 37, 209]. Exceptions to this include decreased responses in elite athletes after performing extreme bouts of exercise such as a marathon and high intensity intervals [230, 237]. Taken together these findings suggest that independent of fatness and sex, fitness may protect against acute exercise induced decrements in vascular function except in extreme cases, and even then the effects are very transient. Thus, timing of the post exercise measure (i.e. FMD) is also a critical consideration.

In general studies that assessed vascular function immediately following acute exercise found a decrease in vascular function, while studies that delayed measurement were more likely to find no effect or an increase in vascular function. Unfortunately a limited number of studies have taken serial measures of vascular measures to elucidate a time response. However there does appear to be a biphasic response, at least with aerobic exercise. A single bout of exercise mimicking high intensity intervals has been shown to decrease acetylcholine induced dilation in abdominal aorta sections rats obtained from female rats immediately following the acute exercise [238]. Furthermore, the dilatory response could be rescued with administration of exogenous SOD indicating a role of excessive oxidative stress. Interestingly acute exercise improved acetylcholine induced dilation at 12 and 24 hours post exercise before vessel sensitivity returned to normal sensitivity at 48 hours. How the *ex vivo* vessels underwent a biphasic response is beyond the scope of this review but it is notable that the vessels exhibited a blunted response compared to vessels

obtained from rats at rest and that oxidative stress seemed to be the primary mediator of dysfunction as indicated the rescue with SOD. Rognmo et al [230] also demonstrated a biphasic FMD response to acute exercise. A decrease in brachial artery FMD was reported one hour following high intensity bicycle intervals in elite endurance trained male athletes. In a matched "control" group of recreationally active athletes, there was an increase in FMD one hour post-exercise. However, both groups demonstrated an increased FMD response at 24 hours post exercise compared to the baseline measure pre-acute exercise. The results from this population are likely not generalizable to the regular sedentary and recreationally active individuals that make up the population at large. The absolute amount of exercise needed to elicit the same relative workload in elite athletes may have evoked excessive oxidative stress in the elite athletes. Lastly, it should be noted that acute resistance exercise induced decrements in vascular function may be longer lasting. Franklin et al [29] showed reductions in brachial artery FMD persisting up to 24 hours.

As noted, exercise intensity is also a determinant of the vascular response to acute exercise. Lower intensity exercise is more likely to have no effect or improve post-acute exercise vascular function whereas higher intensity bouts are more likely to elicit a decrease in vascular function. Harvey et al [239] is an exception in that brachial artery FMD was increased after a higher intensity bout of exercise. However, the FMD was taken one hour following exercise. Tyldum et al [236] also found that an acute bout of both continuous moderate intensity exercise and high intensity interval exercise (using bicycling) increased brachial artery FMD. In a study of the effects of a marathon on vascular function Dawson et al [237] found that running the marathon resulted in a depression in femoral, but not brachial artery FMD. While this study is interesting it is hard to compare to others considering the extreme training status of the athletes, the fact that baseline

FMD was obtained the before the race, the duration of the exercise stimulus, and that the study could not feasibly be as well controlled as the other trials discussed.

Johnson et al [235] had participants perform 20 minutes of low intensity bicycling (90 Watts) and found that the acute exercise increased FMD. However cuff occlusion on the contralateral arm during cycling increased retrograde shear stress and decreased FMD. Retrograde shear evoked excess oxidative stress, as indicated by the finding that vitamin C pre-treatment prevented a post-exercise decrease in FMD in the cuffed limb; and augmented an increase in FMD in the control arm compared to baseline.

Tinken et al [22] found an increased FMD response when subjected participants to exercise. The exercise intensity was adjusted to match shear rate to the shear rate obtained during 40±1°C forearm heating and started out at 1-2 kg for hand gripping and 80 watts for bicycling, both of which would be considered low intensity exercise. Mills et al [229] appears to be the only study that used children. Similar to the other studies reviewed children did not demonstrate a decrease in FMD following low intensity exergaming but did experience a decrease in FMD following high intensity exergaming.

Special consideration should be given to resistance exercise as all the studies highlighted here found a decrease in vascular function following an acute bout of resistance exercise. High blood pressure during exercise is emerging as the most likely candidate in regards to post-acute exercise endothelium dysfunction in resistance exercise models. McKelvie found an increase in total peripheral resistance with lower body resistance exercise (unilateral leg press) in a crossover study comparing hemodynamics during resistance exercise and lowed body cycling. This finding explains why systolic blood pressure was increased to a similar extent with cycling despite cycling evoking a much greater cardiac output [240]. Our group has shown repeatedly that resistance

exercise is associated with an increase in blood pressure [27-29, 34-37, 209]. Gonzales et al [226] also used resistance exercise, but they used an acute bout of hand grip exercise. Hand gripping at faster velocities was associated with a greater concomitant increase in retrograde shear stress compared to slower contractions. Interestingly, slow contractions were associated with greater increases in blood pressure during exercise and only slow contractions were associated with a post exercise decrease in brachial artery FMD. This finding suggests increased blood pressure during exercise supersedes the effect of retrograde shear stress, at least in regard to brachial artery FMD. Gori et al [225] also used hand grip exercise but at a higher intensity. Subjects in this study performed cyclic bouts of isometric hand gripping (100% 1-RM) and post-acute exercise brachial artery FMD was impaired. While this study did not report blood pressure responses, it should be noted that previous studies indicate that concentric contractions are associated with an increased ET-1 and blood pressure post-acute exercise [241]. The findings from these studies demonstrate high blood pressure during resistance exercise is a modulator of post-exercise endothelium function.

In vitro models of high pressure inducing endothelium dysfunction include findings from human saphenous vein segments and internal thoracic artery segments. Isolated vessels subjected to high pressure (170 mmHg) demonstrated reduced stimulated $NO^{\cdot}$ release and increased immunocyte adhesion [242]. In an *in situ* model using open-chest anesthetized dogs subjecting the left anterior descending coronary artery to 30 minutes of hypertension (200 mmHg) augmented endothelium dependent constrictor responses to serotonin [243]. Even exposures to as little as one to five minutes of hypertension evoked the increased constrictor sensitivity for up to two and half hours. Mouse carotid arteries subjected to 30 minutes of high pressure (180 mmHg) have been shown to demonstrate impaired vasodilation to acetylcholine and increased O_2^- production, both

of which were rescued via inhibition of NOX [244]. A limited number of studies have translated these findings to humans as well.

Millgard et al [245] investigated if acute exposure to hypertensive levels of blood pressure impairs endothelium function in normotensive participants. A one hour norepinephrine infusion was used to elevate blood pressure and was administered prior to and following forearm blood flow assessment. Increased diastolic blood pressure (≥95 mmHg) elicited a decrease in forearm blood flow. In a subset of the participants dilation to methacholine was also assessed via infusion and elevated blood pressure also impaired methacholine-induced dilation. A major confounder to this study was the use of norepinephrine, which make the findings more translatable to a state of heightened sympathetic nervous system activity rather than high pressure, per se. This was further confirmed by findings of reduced dilation to the $NO^{\cdot}$ donor sodium nitroprusside indicating the norepinephrine acted beyond the endothelium. Durand et al [36] used ex vivo isolated blood vessels from human subjects in order to elucidate the effect of high blood pressure alone on acetylcholine induced dilation. Interestingly, high pressure exposure (150 mmHg) mimicked the effects of acute resistance exercise and was not suppressed in resistance trained subjects. The maintained post-acute exercise vasodilation in resistance exercisers was H_2O_2 mediated dilation in contrast to $NO^{\cdot}$ mediated dilation at rest. Furthermore blockade of local RAS with Losartan restored acetylcholine induced dilation in sedentary participant's resistance arteries. The pressures used to in induce vascular dysfunction in the animal and human models described here are well within the elevated blood pressure levels experienced during resistance exercise [29, 33, 34, 240] and during hypertensive responses to aerobic exercise [246]. Taken together, these findings suggest that transient exposure to high blood during acute exercise, especially during resistance exercise, may predispose the vasculature to impaired vasodilator function.

Another proposed mechanism for impaired endothelium function following acute exercise is retrograde shear stress. The findings in this area are equivocal. It has been known for some time that antegrade shear stress elicits endothelium-dependent vasodilation [247]. It is also well known that oscillatory shear and retrograde shear can have detrimental effects such as increased Endothelin-1 expression, however, this type of shear also increases phosphorylation of eNOS S1177 [248]. The role of retrograde shear during exercise is still not entirely clear.

Using bilateral forearm heating, recumbent leg cycling, and bilateral handgrip exercise to increase blood flow and shear, Tinken et al [22] found cuffing one of the limbs to prevent increases in antegrade shear stress prevented an increase in brachial artery FMD as seen in the un-cuffed limb. Furthermore using a cuff to attenuate hand grip exercise induced increases in shear stress in humans has been shown to prevent improvements in brachial artery FMD witnessed in un-cuffed limbs following an eight week exercise intervention in young men. [21] In contrast Llewellyn et al [228] found a decrease post-acute exercise brachial artery FMD and baseline shear rate was increased immediately after treadmill running. However total shear exposure during the reactive hyperemic response following occlusion during FMD was not changed. Thus, the authors postulate that the increase in shear during exercise desensitized the artery to the shear stimulus during reactive hyperemia. Jones et al [231] found that time of day affected the occurrence of a post-exercise reduction in FMD despite no differences in shear patterns in morning versus evening exercise (intermittent cycling at 70% VO_{2Max}).

The shear associated with different modalities differs and some exercise create an oscillatory like shear pattern by increasing retrograde shear but this does not result in reduced FMD. For example rhythmic lower limb exercise such as walking and bicycling result in an increase in brachial artery antegrade blood flow and shear rate during systole directly followed by

an increased (relative to rest) retrograde flow and shear rate during diastole. Leg kicking results in antegrade blood flow and shear rate during systole but is not followed by retrograde flow and shear rate during diastole [201]. However both walking and bicycling are not associated with decreased FMD post-acute exercise. Furthermore Gonzales et found faster hand grip contractions were associated with greater retrograde:antegrade shear compared to slow contractions, yet slow contractions induced impaired brachial artery FMD while fast contractions had no effect [226]. The evidence for antegrade:retrograde shear impacting post-acute exercise endothelium are not very compelling at this point.

An increase in sympathetic activity may be a primary mediator of impaired endothelium function following acute bouts of exercise. At onset of exercise, there is a requisite increase sympathetic activity that correlates with the intensity of the exercise [249, 250]. This a major component in modulating the cardiovascular, hormonal, and metabolic responses needed to undertake exercise. Furthermore, manipulation of sympathetic outflow alone has been shown to alter FMD. Lower body negative pressure is highly validated technique to elicit sympathetic stimulation by selectively unloading the cardiac baroreflex [251]. Lower body negative pressure increases muscle sympathetic nerve activity in the peroneal and radial nerves [251, 252] and increases norepinephrine spillover into the plasma [253].

In a study of sixteen healthy volunteers, participants had their brachial artery FMD assessed at rest and after exposure to lower body negative pressure until they experienced a 15% increase in heart rate compared to their resting value or negative pressure reached −20 mmHg [254]. A subset of eight participant performed this same protocol twice, once with intra-arterial infusion of the alpha-adrenergic blocker phentolamine. Brachial artery FMD was suppressed following sympathetic stimulation and rescued by alpha-adrenergic blockade with phentolamine.

Sympathetic stimulation had no effect on nitroglycerin mediated dilation suggesting the effects were confined to the endothelium. At −20 mm Hg, several studies have shown an increase in sympathetic activity without concomitant increases in cardiac output or blood pressure indicating that no other confounders could have influenced the FMD responses. [251, 252, 255, 256] Although lower body pressure has been shown to increase forearm vascular resistance suggesting that the effects may influence the microcirculation as well [253].

Dyson et al [257] designed a study to assess the effects of various means of inducing sympathetic activation on brachial artery FMD. Lower body negative pressure at -30 mmHg did not influence FMD, neither did a mental arithmetic challenge, while the cold pressor test impaired FMD, and the muscle chemoreflex protocol improved FMD. These results refute the previous findings of lower body negative pressure but several studies suggesting mental stress challenges may impair FMD [258-260]. Interestingly, while heart rate was significantly increased during the muscle chemoreflex and mental arithmetic challenges it was unaffected by the lower body negative pressure. Perhaps differences in the subjects studied or methodology employed can explain the conflicting findings between this study and the one described above. The results of the latter study suggest that concluding sympathetic activation impairs FMD may be a generalization and that stimulus specificity may be an important consideration.

In a study designed specifically to address the impact of sympathetic nervous system activity on post-exercise brachial artery FMD Atkinson et al [261] found that 30 minutes of bicycling at 75% maximum heart rate impaired FMD, taken immediately post exercise in ten healthy male participants. However, when participants took the α1-adrenoreceptor blocker prazosin the decrease in post exercise FMD was prevented. An interesting note is that under both conditions retrograde shear rate was elevated and to a similar extent. However mean arterial blood

pressure was increased ~40 mmHg in the control condition and only ~30 mmHg with the prazosin. This was not a significant reduction but with only 10 participants included in the analysis, the study was likely not powered to detect this difference and thus the reduction in blood pressure may have played a role in the improved FMD response nonetheless. An interesting future up study would be to repeat the same study in fit vs. unfit participants to elucidate if sympathetic blockade elicits a response in trained individuals.

Studies indicate individuals with a higher level of fitness are less susceptible to post exercise reductions. However cross sectional studies have not shown reduced muscle sympathetic nerve activity in endurance trained individuals compared it sedentary counterparts [262, 263]. However it could be that sedentary individuals are less able to overcome acute exercise. Decreased blood pressure and improvement in CVD risk are associated with modulation of sympathetic nervous system activity [264] and regular exorcisers demonstrate both of these traits. In summary there does appear to be a strong role for increased sympathetic output during acute exercise in mediating a potential decrease in endothelium function.

Another plausible factor for an acute reduction in endothelium function wth acute bouts of exercise is elevated free fatty acids (FFAs) [265, 266]. It is well known that insulin's effect on the endothelium is mediated through its own receptor resulting in increased release of $NO^{\cdot}$. Indirect evidence for the role of elevated FFA in impairing endothelium function comes from studies demonstrating impaired endothelium function in insulin-resistant conditions such as obesity, Type II diabetes mellitus [184, 185, 267], and in individuals who eat a habitualy high fat diet [268]. Further studies have shown postprandial FMD is decreased following high fat feeding [269].

Elevating circulating free fatty acids (FFAs) can impair endothelium function using both exogenous and endogenous means. Steinberg et al [267] measured leg blood flow at the common

femoral artery with graded intrafemoral artery infusions of the endothelium-dependent vasodilator methacholine in the baseline condition and after elevating FFAs exogenously via a two hour infusion of intralipid plus heparin or endogenously by an infusion of somatostatin to inhibit insulin secretion. Both means increased FFAs and both means were also found to reduce blood flow in response to methacholine by ~20%. There was no change in blood flow following increased FFAs in response to endothelium-independent vasodilator sodium nitroprusside (SNP). To further illustrate the point that the effects of somatostatin were caused by insulinopenia, an experiment was done with insulin co-infused with somatostatin. The co-infusion of insulin suppressed the increase in FFAs and restored dilation to methacholine to baseline levels.

Kim et al [270] treated of bovine aortic endothelial cells with with 100 μM palmitic acid to demonstrate that increaed levels of FFA could elicit impaired insulin induced eNOS signaling through the IKKβ/NFkβ pathway. Specifically insulin stimulated phosphorylation at S 1179 was decreased as was Akt phosphorylation at S 473. Furthermore a DAF-2 DA fluorescent assay was used to assess $NO^{\cdot}$ production and $NO^{\cdot}$ production was also reduced. When were cells transfected with a dominant-negative IKKβ protein the effects of elevated FFAs were blocked. Thus an elevation in FFAs during exercise may likely contribute to impaired endothelium function post-acute exercise.

The potential mediators of impaired endothelium function likely work in concert. For example AT_1r blockers which are thought to reduce the actions of RAS also reduce inflammatory measures such as TNFα and CRP indicating a link between RAS and inflammation[271]. In addition elevation in FFA is associated with an increase in inflammation and oxidative stress [272]. Furthermore animal models demonstrate that RAS augments activity of sympathetic nervous system [273, 274]. Taken together these findings support a model whereby an increase in free fatty

acids, inflammation, sympathetic nervous system activity, high pressure, and oxidative stress may all influence the endothelium independently, and in tandem to induce vascular dysfunction.

An important methodological note to make here is that most of the studies assessing post-acute exercise vascular function described here used ultrasound assessment of brachial artery FMD and *ex vivo* microvessel preparations. Other studies using means such as strain gauge plethysmography have also contribute to this body literature. However, they were not reviewed here. While brachial artery FMD is a well-established technique with prognostic value, the technique is limited in its standalone ability to provide mechanistic insights. Using isolated human adipose microvessels allows for extensive use of pharmacology as the vessels are *ex vivo*. This is important as more mechanistic studies will be needed in the future in order to determine how fit populations maintain vascular function following post-acute exercise. In addition the resistance exercise studies to date have used brachial artery FMD, however the results Dawson et al [237] in running indicates that using the superficial femoral artery or popliteal may be a more sensitive measure in these studies.

Table 1 Studies investigating the effect of acute exercise on vascular function

Study	Measure	Population	Acute Exercise	Time point	Outcome
Bailey et al 2012[224]	Brachial artery FMD	13 moderately trained young males	Maximal treadmill running test + 5 km running time trial	Immediately post exercise	Decrease; Prevented by ischemic preconditioning
Cosio-Lima et al 2006 **[233]**	Brachial artery FMD	11 healthy middle aged individuals (8F/3M) 11 renal transplant recipients (8F/3M)	30 minutes "somewhat hard" treadmill walking	Immediately post exercise	Increased No effect
Durand et al 2015**[36]**	Brachial artery FMD resistance artery ACh-induced dilation	21 sedentary young adults (13F/8M) 33 heathy young exercisers (10F/23M)	4 sets of 10 repetitions bilateral leg press (intensity not specified, ~80% 1-RM based on description)	FMD immediately after; followed by biopsy for resistance arteries (~30 minutes)	Decreased No effect
Franklin et al 2014**[29]**	Brachial artery FMD	26 young sedentary adults (28F/8M)	8 sets of 10 repetitions bilateral leg press at 80% measured 1-RM	60 minutes post exercise	Decreased; prevented by massage therapy
Franklin et al 2014**[28]**	Brachial artery FMD	8 young healthy women (BMI <25) 9 obese young women (BMI 30-40)	3–4 sets of 10 repetitions bilateral leg press at 80% estimated 1-RM	Immediately post exercise (≤15 minutes)	Decreased Decreased

Franklin et al 2015**[27]**	Brachial artery FMD	18 young overweight women (BMI 30-40)	8 sets of 10 repetitions bilateral leg press at 80% estimated 1-RM	Immediately post exercise (≤15 minutes)	Decreased; 8 weeks of circuit resistance training in a subset of 10 participants prevented the decrease
Gonzales et al 2011**[226]**	Brachial artery FMD	14 young healthy adults(8F/6M)	30 minutes of hand gripping at: 0.5m/s ("fast") 0.2m/s ("slow")	30 minutes post exercise	 No effect Decreased
Gori et al 2010**[225]**	Brachial artery FMD	12 young healthy adults(5F/7M)	Four minutes of rhythmic isometric hand gripping; no cadence	Immediately post exercise	Decreased
Harris et al 2008**[38]**	Brachial artery FMD	16 overweight men: 8 fit (VO_{2Max} ~35) 8 unfit (VO_{2Max} ~27)	45 minutes of low (25% VO_{2Max}), moderate (50% VO_{2Max}), or high (75% VO_{2Max}) intensity treadmill walking	60 minutes post exercise	Independent of exercise intensity Increased Decreased
Harvey et al 2005**[239]**	Brachial artery FMD	13 postmenopausal women	45 minutes of treadmill walking at 75% VO_2Max	60 minutes post exercise	Increased
Hwang et al 2012**[30]**	Brachial artery FMD	74 young healthy adults (39F/35M)	Maximal treadmill test (Bruce protocol)	Immediately post exercise	Decreased in Females No effect in males No effect in exercisers (>3d/wk)
Johnson et al 2012**[235]**	Brachial artery FMD	12 young males healthy	20 minutes of bicycling at 20 watts	Immediately post exercise	Increased

Jones et al 2010**[231]**	Brachial artery FMD	12 young healthy males	Three 10 minute bouts of cycling at 70% VO_{2Max} performed at Morning (8 a.m.) Evening (4 p.m.)	20 to 30 minutes post exercise	 No effect Decrease
Jurva et al 2006**[34]**	Brachial artery FMD	14 young resistance exercise trained adults (5F/9M) 16 young sedentary adults (5F/11M)	2-3 sets of 6 to 8 repetitions bilateral leg press (intensity not specified, ~85% 1-RM based on description)	Immediately post exercise	Decrease No effect
Llewellyn et al 2012**[228]**	Brachial artery FMD	15 young healthy adults(8F/7M)	30 minutes treadmill running at the velocity used to obtain 60% VO_{2Max} during a maximal test	Immediately post exercise	Decreased
McGowan et al 2006**[227]**	Brachial artery FMD	20 hypertensive adults (F/M not provided)	4 sets of 2 minute hand grip exercise at 30% MVC	Immediately post exercise	Decreased
McKelvie et al 1995**[240]**	Basic hemodynamics-Blood pressure, EKG	10 males with congestive heart failure	5 minutes of bicycling at 50% peak power output during max test 2 sets of 10 repetitions unilateral leg press at 70% estimated 1-RM	Continuously and immediately post exercise	Both interventions increased SBP. Cycling increased hear rate and rate pressure product to a greater extent. Resistance exercise increased total peripheral resistance to a greater extent.

Mills et al 2013**[229]**	Brachial artery FMD	15 healthy children (7F/8M)	Low intensity exergaming (~105 bpm) High intensity exergaming (~140bpm)	Immediately post exercise	No effect Decreased
Phillips et al 2011**[37]**	Brachial artery FMD	13 young sedentary males 13 resistance exercise trained males 13 aerobic exercise trained males 14 cross trained males	2-3 sets of 6 to 8 repetitions bilateral leg press (intensity not specified, ~85% 1-RM based on description)	Immediately post exercise	Decreased Increased Increased No effect
Rognmo et al 2008**[230]**	Brachial artery FMD	10 male recreational athletes (VO_{2Max} ~75) 10 male elite athletes (VO_{2Max} ~75)	Interval running 5 x sets of 5 minutes at 90% of maximal heart rate	One hour	Increased Decreased
Thijssen et al 2006**[232]**	Superficial femoral artery FMD	10 young healthy males 8 elderly healthy males	Incremental maximal cycling test	Immediately post exercise	No effect No effect

Tyldum et al 2009**[236]**	Brachial artery FMD	8 young healthy males	47 minutes of treadmill running at 60-70% max HR 4 sets 4 minutes treadmill running at 85-95% max HR	Immediately post exercise	Increased Increased
Tinken et al 2009**[22]**	Brachial artery FMD	10 young recreationally active male	Handgrip exercise at 30 contractions/min for 30-min Recumbent leg cycling 30-minutes at 60–70 rpm (>80W)	Immediately post exercise	Increased Increased
Varady et al 2010**[209]**	Brachial artery FMD	10 young sedentary males 10 resistance exercise trained males 12 aerobic exercise trained males 11 cross trained males		Immediately post exercise (≤15 minutes)	Decreased Increased Increased Increased

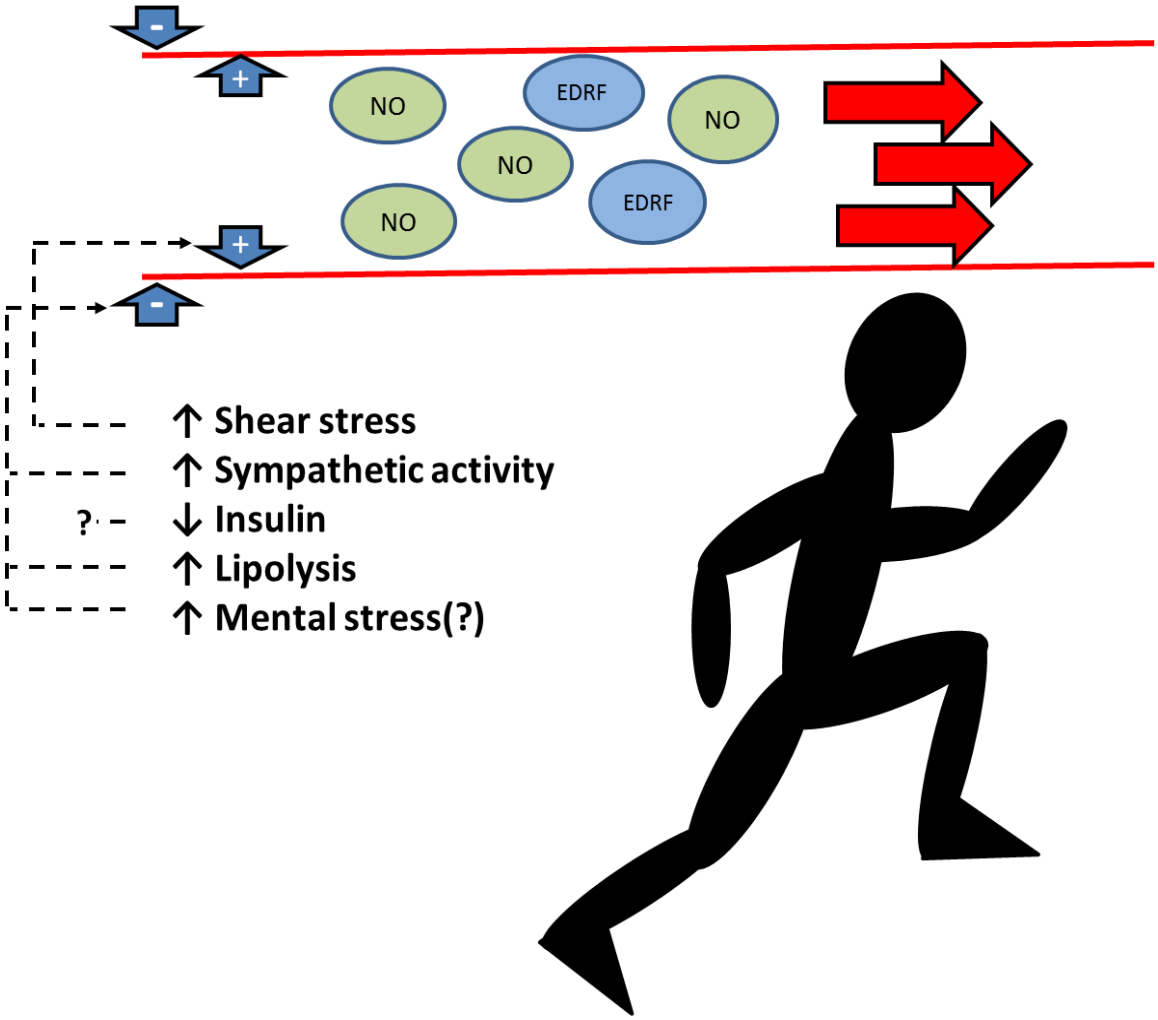

Figure 8 Exercise modulators of peripheral vascular function.

An increase in shear stress contributes to increased NO· production and facilitates vasodilation. There appears to be a strong role for heightened sympathetic nervous system activity and an increase in free fatty acids via lipolysis for reducing vasodilation post-acute exercise. The mental stress component of high intensity exercise can be ruled out and ay contribute a post-acute exercise decrease in vascular dysfunction. The role of insulin fluctuation during acute exercise on vascular function has not been studied.

II. G Summary:

In summary, while the literature supports that exercise is beneficial, acute exercise can potentially be detrimental depending on the intensity of the exercise and the characteristics of the individual partaking in the activity. [24, 25] To be clear higher intensity exercise seems to cause transient vascular dysfunction at least as measured by brachial FMD and isolated resistance artery studies immediately after exercise. Furthermore, there are a limited number of studies that have investigated resistance exercise as the acute exercise stimulus. [7, 27-29, 34, 36, 37, 226] and there is little published work on the effects of acute physical exertion on endothelium function in obesity. Obesity is an epidemic with negative vascular consequences and there is a need for studies elucidating the effect of acute strenuous exertion on the microcirculation and further providing mechanistic insights into the mediators of post- acute strenuous exertion vascular dysfunction or preserved function in this population. These individuals may be at even greater risk of post-acute exertion vascular dysfunction and make up a large proportion of the population. It appears that the key mediators of acute strenuous exertion vascular dysfunction include increased sympathetic activity, exposure to high blood pressure, inflammation, and potentially elevated free fatty acids. The focus of our laboratory has been high pressure as the key mediator of acute resistance exercise induced vascular dysfunction. This type of exercise has been shown to cause large increases in systolic blood pressure [28, 33, 34, 37]. Furthermore there is evidence to suggest that high pressure exposure, independent from acute exercise, elicits endothelium dysfunction [41, 49, 245] and this may be mediated by activation of RAS NOX II, and subsequent oxidative stress [36, 275]

The purpose of the following experiments presented here were to test if regular aerobic exercise prevent acute high exertion induced vascular dysfunction in overweight and obese individuals and investigate potential mediators of post- acute strenuous exertion vascular dysfunction or preserved function. Furthermore, we used a murine and cell model to gain further

mechanistic insights into high pressure induced vascular dysfunction because high pressure appears to be the key mediator of post- acute strenuous exertion vascular dysfunction. We hypothesized regular exercise in microvessels and shear tress exposure in cells would confer vascular conditioning that would protect the endothelium against high pressure decreasing pro-oxidant enzyme expression and increasing anti-oxidant enzyme expression.

Chapter III: Improved Arterial Flow Mediated Dilation after Exertion involves Hydrogen Peroxide in Overweight and Obese adults following Aerobic Exercise Training.

* Parts of this chapter were previously published as Robinson AT, Franklin NC, Norkeviciute E, Bian JT, Babana JC, Szczurek MR, & Phillips SA. Improved arterial flow-mediated dilation after exertion involves hydrogen peroxide in overweight and obese adults following aerobic exercise training. July 2016 –Volume 34 – Issue 7 – p 1309-1316. Wolters Kluwer Health Lippincott Williams & Wilkins©

III. A Introduction

Regular exercise is recommended to combat cardiovascular disease (CVD), the number one cause of death in the United States [276-278]. In addition regular exercise is recommended for the prevention and management of obesity, another epidemic in the United States that is a major risk factor for CVD [16]. However acute strenuous physical exertion increases the risk of cardiovascular events in sedentary adults [24, 25]. In line with this observation, previous studies indicate that large artery endothelium function is reduced for periods up to 24 hours following bouts of strenuous exertion depending on the population being investigated. [26, 28, 34, 37, 38, 115] Several of these studies have implicated transient exposure to high blood pressure as a potential explanation for exertion induced decrements in arterial function [28, 29, 34, 37]. Indeed it has been found that during resistance exercise systolic blood pressure can rise to over 300 mmHg and diastolic blood pressure can rise to over 150 mmHg [33]. Many other studies have failed to replicate such large increases but there is an agreement that blood pressure does increase during acute strenuous physical exertion.

Cross-sectional studies indicate that the presence of a post-exercise decrease in brachial artery flow mediated dilation (FMD) [37, 38] does not occur in exercise-trained populations

indicating fitness protects against exertion induced decrement in large artery function. Furthermore, recent work from our laboratory indicates that that these trends are mirrored in the microcirculation. Isolated microvessels obtained from sedentary individuals exhibit impaired acetylcholine induced dilation (AChID) following and acute bout of bilateral leg press or exposure to elevated intraluminal pressure (150mmHg) following cannulation [36, 41, 157]. In contrast, microvessels obtained from regular exercisers exhibit preserved AChID following acute strenuous physical exertion or exposure to high intraluminal pressure [36]. Furthermore, preserved AChID following acute strenuous physical exertion or exposure to elevated intraluminal pressure appears to be mediated via H_2O_2 whereas resting microvessel AChID is predominately $NO^{\cdot}$ dependent. That both microvessel and brachial artery FMD are impaired following acute strenuous exertion or high pressure exposure in sedentary individuals but preserved in exercisers is not overtly surprising as recent findings suggest that endothelium dilations of *ex vivo* microvessel (in vessels obtained during rest) and resting brachial artery FMD are correlated [279].

What remains to be studied is whether higher fitness obtained via regular exercise training can prevent large artery and microvessel endothelium function in response to acute exertion. Furthermore, the response of the microcirculation in response to acute strenuous physical exertion has not been studied in an obese and overweight population. We presumed that this population is at risk as both sedentary lifestyle and the manifestations of being overweight/obese are both independent risk factors for cardiovascular disease [280-282]. Therefore, we sought to determine if eight weeks of moderate-intensity aerobic exercise (AT) could prevent physical exertion-induced large artery and microvessel dysfunction in sedentary overweight and obese individuals. Furthermore, we used pharmacological agents to block the actions of $NO^{\cdot}$ and H_2O_2 to gain mechanistic insights into the regulation of endothelium dependent vasodilator function in

microvessels. We hypothesized that following eight weeks of aerobic exercise: 1) brachial artery and microvascular dysfunction following acute exertion will be prevented, and 2) hydrogen peroxide would contribute to the preserved microvessel flow induced dilation following acute physical exertion in obese adults.

III. B Methods

Study Design

Twenty-five previously sedentary overweight and obese individuals (BMI: 25-40 kg/m^2) participated in this study. Volunteers were assigned to either AT (13 participants) intervention or control (CON; 12 participants) and 21 individuals (84%) completed all the requirements of the study. However, the data from two female participants were excluded as they were post-menopausal. Final analyses included data from 19 participants (10 AT and 9 CON). Subject characteristics can be found in Table 1. Participants were matched for age, anthropometrics, and fitness level. Exclusion criterion included: history of cardiovascular disease and related morbidities, diabetes mellitus, thyroid dysfunction, cancer, pregnancy, aged $\leq$ 18 or $\geq$ 55, and smoking. In order to determine if study eligibility was met, individuals were initially pre-screened via telephone interview. Individuals who met pre-screening inclusion criteria were invited for an in-person screening consisting of a physical examination, completion of medical and exercise history questionnaires, and followed by providing written informed consent before participation in study related activities. Inclusion criteria for the study consisted of: no history of cardiovascular disease (CVD), diabetes mellitus, thyroid dysfunction, cancer, not currently or recently pregnant or lactating, and non-smoking (at least 6 months prior to enrollment in the study). Individuals also had to be previously sedentary (less than 150 minutes of physical activity per week) and overweight or obese (BMI $\geq$ 25 kg/m^2). The study protocol was approved by the Institutional

Review Board at the University of Illinois at Chicago (UIC). All participants provided informed consent before participation. Visits were conducted at UIC's Clinical Interface Core's Clinical Research Center (CRC) and Integrative Physiology Laboratory (IPL).

Aerobic Exercise Intervention

All participants underwent assessment for aerobic capacity at week zero and week eight at UIC's IPL. Following baseline assessments, participants assigned to CON were instructed to not increase their normal physical activity level. Participants in the AT group commenced their eight week exercise intervention. For assessment of aerobic capacity a graded treadmill test was performed to exhaustion as previously described [283] at week zero and week eight. Another assessment of aerobic capacity was performed at week four (study mid-point) in order to re-calibrate exercise intensity prescription. The AT intervention was designed to meet established AHA physical activity guidelines for adults. Participants reported for training sessions on non-consecutive days, three days per week for eight weeks. All sessions were supervised by a qualified fitness professional. Each session began with a warm-up consisting of dynamic movements including lunges and side lying single leg raises in order to elevate body temperature and taking the lower body joints through a full range of motion, in order to prevent injury or discomfort from the repetitive sagittal plane-only movement that our treadmill based intervention entailed [284]. Treadmill exercise commenced with a slow walk for 3 minutes followed by an increase in speed and/or incline to elicit an average heart rate of 75% max heart rate using Karvonen method . Re-assessment of aerobic capacity and subsequent exercise prescription was performed at week 4. Treadmill exercise progressed in duration from 30 minutes in initial weeks to 45 minutes by weeks

seven and eight. Each session ended with a 5-minute cool-down which consisted of slow treadmill walking and static stretching.

Acute Exertion Protocol with Leg Press Exercise

To investigate the effects of acute strenuous physical exertion on vasodilation participants underwent a bout of leg press during visits at week zero and week eight as previously described [28]. Briefly, the exercise protocol consisted of bilateral lever based leg press (Hoist HD-1610 Selectorized Leg Press; Hoist Fitness Systems; San Diego, California). Participants were familiarized with the leg press machine via performing a warm up of one to two sets of 10 repetitions at a perceived intensity of approximately 30 to 40% of one-repetition maximum (1-RM). Participants then performed 6 to 8 sets of 10 repetitions on a 2 second: 2 second cadence at a perceived intensity of approximately 70 to 80% of 1-RM. A ~two-minute rest period was allotted between each set and subjects were encouraged to drink water ad libitum. Blood pressure, heart rate and the 10-point Borg rating of perceived exertion (RPE) scale were used as indices of intensity after each set. All participants completed a minimum of 6 sets of 10 repetitions. When subjects could not complete a repetition, assistance was given in order to complete the concentric portion of the lift and then completed the eccentric portion of the lift unassisted. These procedures were continued until 10 repetitions were met. Failure typically did not occur until the last 2-3 repetitions of the last two sets of exercise sessions.

Clinical Measures

Resting systolic blood pressure and diastolic blood pressure were measured pre and post intervention using an automated blood pressure device (MassimoSEL, Welch Allyn). Waist

circumference was measured at the level of the umbilicus. Body fat% was determined using dual energy x-ray absorptiometry (DEXA; Lunar iDXA, GE Healthcare) as previously described [285]. Plasma samples were obtained via centrifugation (1600 RCF) of venous blood samples drawn from the antecubital vein into EDTA tubes. Plasma was either measured immediately or frozen at -80°C for subsequent analysis. Total cholesterol, high-density lipoproteins (HDL), low-density lipoproteins (LDL), glucose, and insulin concentration measurements were conducted at Alverno Clinical Laboratories (Crown Point, IN). Tumor Necrosis Factor-α (TNF-α) and C-reactive protein (CRP) were measured using enzyme-linked immunosorbent assay (ELISA; R&D Systems) and Superoxide Dismutase (SOD) via a colorimetric assay kit (Cayman Chemical). Samples were tested by our laboratory in duplicate and coefficients of variation were TNF-α: 4.82%, CRP: 6.76%, and SOD: 6.53%.

Brachial FMD

Brachial artery endothelial function was measured via FMD. Brachial artery FMD guidelines have been previously published [61]. We measured pre- and post- acute bilateral leg press exercise brachial artery FMD as follows: subjects were instructed to lie supine in a climate-controlled room (22–25°C) for 10 minutes to establish a hemodynamic steady state. The brachial artery was imaged in the longitudinal plane using high resolution 2D ultrasonography (Sonosite M Turbo; Seattle, WA). Images were obtained using a high-frequency (7.5 MHz) linear array probe at depth of 30-50mm. The probe was placed two to eight cm proximal to the antecubital fossa. Baseline blood velocity was recorded for ~10 cardiac cycles in Doppler mode at an insonation angle of 55° and baseline diameter images were then recorded for one minute at 5 frames per second followed by forearm blood occlusion using a rapid inflation forearm occlusion cuff (Hokanson) inflated to 200-250 mmHg for 5 minutes. The cuff was then rapidly deflated to induce a subsequent reactive hyperemia (RH). Cuff placement was ≤ 3 cm distal to the antecubital

fossa. Doppler mode was used for measurements of peak hyperemic blood velocity during the first 6-8 cardiac cycles following cuff release before switching back to B-mode for 2D ultrasound imaging of brachial artery diameter for the remaining 3 minutes of the data collection period. The largest recorded diameter throughout the 3 minute post occlusion collection period was used to represent peak diameter. RH velocity and diameter were used to calculate peak shear rate (SR_{peak}) using the following equation: $8 \times \mu \times V_{max}/D_{baseline}$, where μ is blood viscosity (0.035 dyne $\times$ s/cm^2), V_{max} is peak RH velocity, and $D_{baseline}$ is baseline diameter. Baseline shear rate was calculated in the same fashion as SR_{peak} except for using baseline velocity and diameter. BAFMD is expressed as a percent increase in diameter from baseline and calculated as:

FMD(%) = (peak RH diameter − baseline diameter)/baseline diameter x 100.

Brachial artery FMD adjusted for shear stress was equated as FMD/SR_{peak}. Adjusted brachial artery FMD were then log transformed to allow for parametric testing. Nitroglycerin (NTG; 0.4 mg)-induced dilation was also assessed at week zero and week eight in order to confirm any impairments were confined to the endothelium [286]. All image acquisitions were performed by the same investigator. In addition, all measurement analyses were performed by a separate investigator. The coefficient of variation (intraobserver) for our laboratory has been reported previously. The coefficient of variation was 1.5% for brachial artery diameter, 6.3% for FMD, and 3.2% for NTG-induced dilation [287].

Microvessel Flow Mediated Dilation

Microvascular function was assessed as follows. All participants received two fat biopsies on opposite hips over the course of two visits, at rest following an overnight fast (week 7) and

following BLP (week 8). Subcutaneous gluteal adipose tissue was obtained from the proximal, lateral gluteal region following local anesthesia with lidocaine (20 mg/mL). Specimens were placed in cold (4°C) HEPES buffer solution (140mM NaCl, 4mM KCl, 1.2mM $MgCl_2$, 5mM glucose, 10mM HEPES, and 1.2mM $CaCl_2$) and immediately taken to the vascular biology laboratory (1919 W. Taylor St. Chicago, IL) to undergo physiological testing. Microvessels dissected from the adipose were cleaned of fat and connective tissue and prepared for continuous measurement of diameter. Dilation of resistance areties was observed via videomicroscopy as described previously [36, 41, 157, 288].

Briefly in an organ chamber similar to that described by Duling et al [289], vessels were cannulated with glass micropipettes (internal diameters of 30 to 50 μm) filled with cold Krebs solution (123mM NaCl, 4.7mM KCl, 1.2mM $MgSO_4$, 2.5mM $CaCl_2$, 16mM $NaHCO_3$, 26μM EDTA, 11mM glucose, and 1.2mM KH_2PO_4) and continuously perfused at 40 ml/min (MasterFlex pump, Cole Parmer). Both ends of the vessel were secured on the micropipette with surgical ties (10-0 nylon Ethilon monofilament sutures), and the vessel was maintained at an intraluminal pressure of 60 cmH_2O (~44 mmHg) for 30 min before further experimentation. The organ chamber was aerated with a gas mixture of 21% O_2, 5% CO_2, N_2 balanced, and maintained at 37°C via a perfusion system with tubing connected to a thermostat (PC200, Thermo Scientific, NC, USA). Each preparation was set on the stage of an inverted microscope attached to a video camera, video monitor, and a video-measuring device (Boeckeler; model VIA-100).

After intraluminal pressure of 60 cmH_2O was maintained for 30 min, vessels were constricted 30–50% with endothelin-1 (100 to 160 pmol final concentrations). Vessels that did not constrict >30% were discarded. Flow induced dilation was produced generating pressure gradients

of Δ10, Δ20, Δ 40, Δ60, and Δ100 cmH_2O using Krebs-filled reservoirs. Microvessel FMD was measured in the absence and presence of L-N^G-Nitroarginine methyl ester (L-NAME; 10^{-4}M) and Polyethylene glycol Catalase (PEG-Catalase; 500U/ml) which were added in randomized order to the external bathing solution of the organ chamber 30 minutes before pre-constriction with endothelin-1. Maximal diameter of every vessel (endothelium-independent vasodilation) was determined with the $NO^{\cdot}$ donor Papaverine (10^{-4} M) at the end of each experiment. Percent dilation for microvessel FMD was calculated as the percent change from the ET-1 induced pre-constricted diameter relative to the maximal diameter measured at rest prior to ET-1 constriction:

%FMD= (ΔXX cmH_2O diameter – ET-1 pre-constricted diameter)/(Resting diameter (prior to Et-1) – Et-1 pre-constricted diameter)

L-NAME, PEG-Cat and ET-1 were obtained from Sigma-Aldrich Corporation. Chemical reagents for buffer solutions were also purchased from Sigma-Aldrich Corporation and Fisher Scientific.

Microvessel Fluorescence

Flow-induced $NO^{\cdot}$ and H_2O_2 production were measured in microvessels following cannulation and maintenance of 37 °C in a 20 ml aerated organ chambers bathed in Krebs solution chamber at an equilibration pressure of 60 mmH_2O for 30 minutes. $NO^{\cdot}$ production was assessed using $NO^{\cdot}$ detection reagent from Enzo Life Sciences $NO^{\cdot}$ Detection kit (ENZ-51013-200). H_2O_2 production was assessed using 1 µM DCF-DA (2',7'-Dichlorodihydrofluorescein diacetate). After incubation, vessels were exposed to flow via a pressure gradient of Δ60 cmH_2O created by changing the height of the Krebs filled reservoirs, as described above. Prepared vessels were then excised from glass

micropipettes and subsequently mounted on slides. Mounted vessels were then mounted on slides with DAKO fluorescent mounting medium (Dako North America, Inc Carpinteria, CA, USA) and examined via fluorescent microscopy (Eclipse 80i; Nikon, Japan). Fluorescence was detected with 605-nm and 505nm band pass filters for $NO^{\cdot}$ and H_2O_2 detection respectively. Acquired images were analyzed for fluorescence intensity in arbitrary units (AU) using NIH Image J software. All vessels fluorescence was measured three times along different lengths of the vessel using a pre-defined area. Vessel fluorescence was normalized to 2—D vessel diameter and background intensities were subtracted before quantification of fluorescent AU in order to negate auto-fluorescence. [290, 291]

Statistics

All data are expressed as mean ± SE except where otherwise stated. Clinical parameters, resting BAFMD, BAFMD change scores, and fluorescent images were compared using Two-Way ANOVA (time point and group). Resting microvessel FMD and microvessel FMD change scores (pre to post-acute bilateral leg press) were compared using t tests. The effects of pharmacological inhibitors to baseline dilations were compared using Two-way repeated measure ANOVA. ANOVAs were followed by multiple comparisons using the Bonferroni adjustment. Alpha was set at $P< 0.05$. Fluorescence data was analyzed using Paired or Independent samples t-test depending on if vessels were paired or not. Statistics were ran using SPSS 22.0 (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY).

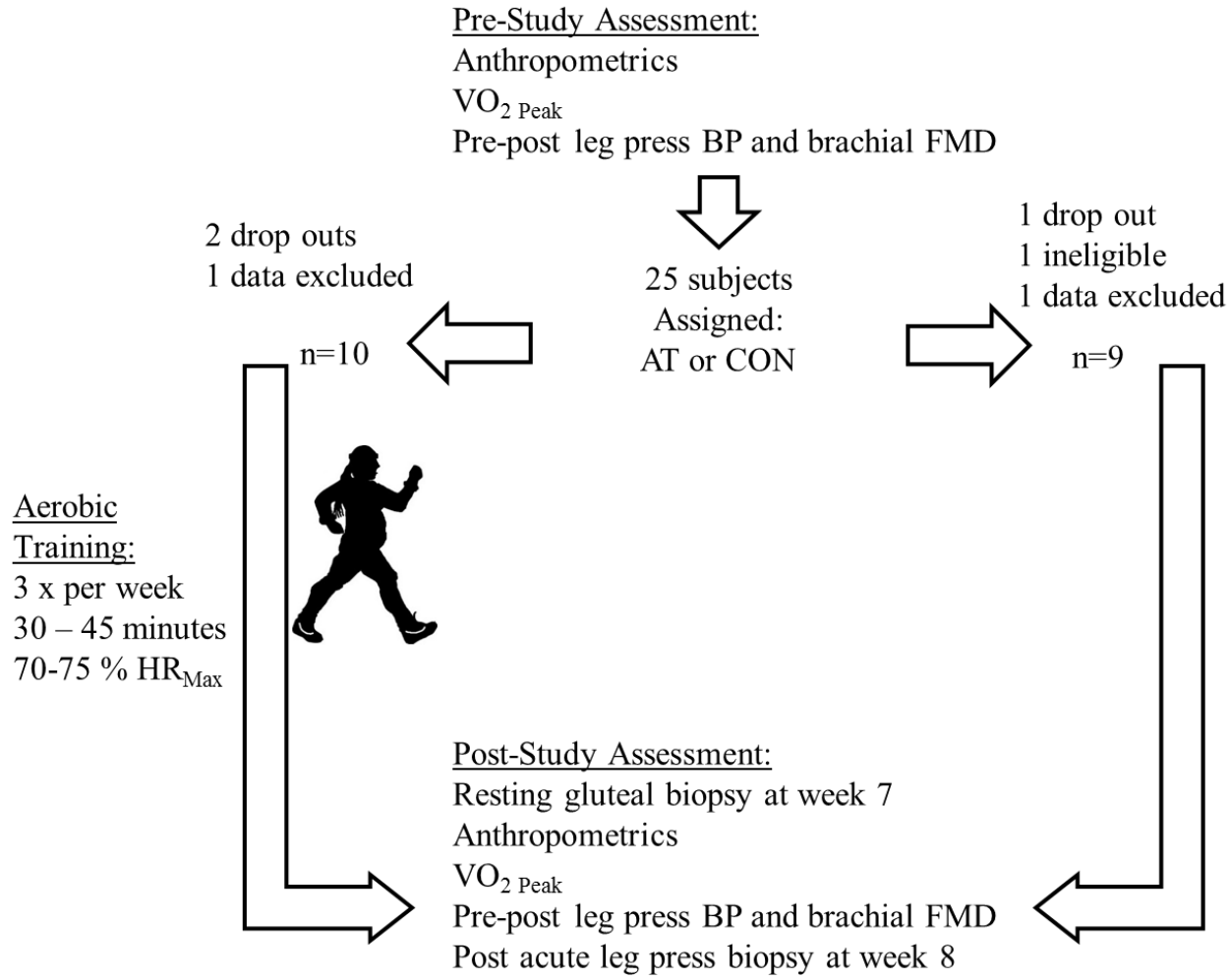

Figure 9 Schematic diagram of the study design and procedures for aim 1.

All participants were screened at baseline (week zero) via questionnaires and physical examination, followed by assessment of brachial artery flow-mediated dilation (FMD) immediately prior to and after performance of an acute bout of bilateral leg press (BLP). During a separate visit, but within the same week subjects underwent assessment of body composition and aerobic fitness ($VO_{2\ Peak}$). At week seven a resting gluteal biopsy was conducting for assessment of microvessel flow mediated dilation (FMD). During follow-up (week eight), participants underwent reassessments of brachial artery FMD pre- and post-acute BLP. Another biopsy was conducted for post-acute BLP assessment of microvessel FMD. Body composition and $VO_{2\ Peak}$ were also reassessed. Evaluation of nitroglycerin-induced dilation was assessed in the after leg press at both week zero and week eight. All data presented as meand ± SE except age which is presented as mean ± SD. Figure used with permission from Journal of Hypertension: July 2016 – Volume 34 – Issue 7 – p 1309-1316. Wolters Kluwer Health Lippincott Williams & Wilkins©

III. C Results

Clinical characteristics of AT and CON participants at weeks zero and eight are presented in Table 3.1. Significant reductions in weight, BMI, Body fat %, waist circumference, diastolic blood pressure, C-reactive protein, and plasma SOD were found following eight weeks of aerobic exercise training ($P < 0.05$). Aerobic fitness as assessed via VO_{2Peak} was significantly improved in the aerobic training participants. There were no changes in the CON group and no statistically significant differences between groups at baseline.

Brachial artery FMD was reduced following acute bilateral leg press before commencing the aerobic training intervention ($\Delta = -2.81 \pm 1.18$ %; $p<0.05$; Fig 11), but was preserved post intervention ($\Delta = +2.48 \pm 1.26$%; Fig 11). The difference in these change scores (AT week 0 vs. week eight) was also significant ($p<0.05$; Fig. 11) Brachial artery FMD in the control group was significantly reduced at week zero and reduced at week eight (although not significantly at week eight; see Fig. 11). There were no significant between or within group differences in resting BAFMD (Fig. 10) or NTG mediated dilation (Fig. 12). When brachial artery FMD was normalized to peak shear rates these trends persisted (see Table 3.2)

Microvessel FMD from vessels obtained at rest was similar in AT and CON. There was no significant difference in diameters of AT and CON microvessels. L-NAME significantly reduced microvessel FMD relative to both baseline in microvessels obtained at rest in both AT and CON participants (Fig. 13 & Fig 15 $P<0.01$). Microvessel FMD following the acute bout of bilateral leg press was lower in vessels from CON participants (Fig. 14) but was preserved in AT participants (Fig. 16). The preserved microvessel FMD in microvessels from AT participants was inhibited by PEG-Cat (Fig 16 $P<0.01$) PEG-Cat also significantly reduced microvessel FMD relative to L-NAME (Fig. 17B $P<0.01$ at 20, 40, 60 and 100 cmH_2O). This trend was the opposite of what

occurred at rest whereby L-NAME significantly reduced microvessel FMD relative to PEG-CAT (Fig. 17A $P<0.01$ at 20, 40, 60 and 100 cmH_2O). This reversal in mediators of dilation did not occur in vessels obtained from the CON group as there were no significant differences between microvessel FMD following PEG-Cat or L-NAME incubation, although both L-NAME and PEG-Cat reduced microvessel FMD relative to basal in post-acute bout of bilateral leg press condition in CON participants. Papaverine induced dilation was similar between groups at both time points (Fig. 18). $NO^{\cdot}$ fluorescence was significantly decreased post-BLP in both AT and CON (Fig. 19).

Nitric Oxide production was similar between CON and AT participants in microvessels obtained during the resting condition. $NO^{\cdot}$ production was reduced in both CON and AT participants following acute bilateral leg press. (Fig. 19). In CON participants H_2O_2 was significantly reduced following acute bilateral leg press, but was significantly increased in AT participants (Fig. 19).

The magnitude of the peak BP response during BLP was not changed from week zero to week eight in AT and CON participants and BP elevations were similar between groups (Fig. 20). The acute and chronic responses to exercise were measured for TNF-α. Eight weeks of AT reduced TNF-α. There was no acute effect of BLP on TNF-α in AT or CON at either weeks zero or eight (Fig. 21).

Table 2 Participants clinical characteristics before and after respective interventions

	Control		Training Effect	Aerobic Training		Training Effect	Baseline Diff
	Pre	Post		Pre	Post	*P*	*P*
Age	28 ± 5		-	34 ± 8		-	0.051
Sex, F/M	(7/2)		-	(7/3)		-	0.719
Height, cm	168 ± 11		-	171 ± 9		-	0.54
Weight, kg	94 ± 17	94 ± 18	0.389	94 ± 14	91 ± 14*	0.015	0.929
BMI, kg/m²	33 ± 6	33 ± 6	0.380	32 ± 5	31 ± 5*	0.042	0.707
Body fat, %	43 ± 11	43 ± 10	0.141	42 ± 8	40 ± 8*	0.011	0.887
Waist circumference, cm	93 ± 11	93 ± 11	0.980	96 ± 12	94 ± 12*	0.017	0.628
Heart Rate, bpm	69 ± 13	71 ± 7	0.312	68 ± 10	62± 5*	0.02	0.844
VO_2, ml/kg/min	33 ± 7	33 ± 7	0.25	32 ± 8	35 ± 9*	0.004	0.648
Systolic BP, mmHg	120 ± 8	123 ± 12	0.312	124 ± 9	120 ± 6	0.148	0.365
Diastolic BP, mmHg	74 ± 9	78 ± 4	0.215	79 ± 9	71 ± 6*	0.038	0.386
Total Cholesterol, mg/dL	179 ± 29	165 ± 15	0.554	179 ± 41	163 ± 30	0.132	0.995
HDL-C, mg/dL	60 ± 9	59 ± 8	0.252	52 ± 13	56 ± 6	0.301	0.125
LDL-C, mg/dL	108 ± 26	92 ± 13	0.525	111 ± 29	100 ± 25	0.176	0.883
Triglycerides, mg/dL	78 ± 25	73 ± 22	0.43	88 ± 33	84 ± 45	0.116	0.646
Glucose, mg/dL	85 ± 6	88 ± 9	0.309	90 ± 12	87 ± 8	0.283	0.248
Superoxide Dismutase, U/mL	3.6 ± 1.3	3.7 ± 2.2	0.706	2.5 ± 0.4	3.0 ± 0.4*	0.011	0.124
C-Reactive Protein, mg/dL	2.3 ± 0.9	2.6 ± 1.1	0.276	3.7 ± 0.9	1.8 ± 0.4*	0.038	0.497

AT participants experienced significant improvement in several markers of health noted by an asterisk and bolded *p* values. *Statistical significance within AT group after eight weeks of training (P <0.05). There were no within group differences after the study for CON

participants. There were no between group differences between AT and CON groups at baseline. Within group differences were compared using paired sample t-tests. Baseline differences were compared using independent sample t-tests. Data presented as mean ± SD. WC = waist circumference, HR = heart rate VO_2 = aerobic capacity, SBP = systolic blood pressure, DBP = diastolic blood pressure, TC = total cholesterol, HDL-C = high density lipoprotein cholesterol, LDL-C = low density lipoprotein cholesterol, TRG = triglycerides, SOD = Superoxide Dismutase, CRP = C-reactive protein. Data are presented as mean ± SD. Table used with permission from Journal of Hypertension: July 2016 –Volume 34 – Issue 7 – p 1309-1316. Wolters Kluwer Health Lippincott Williams & Wilkins©

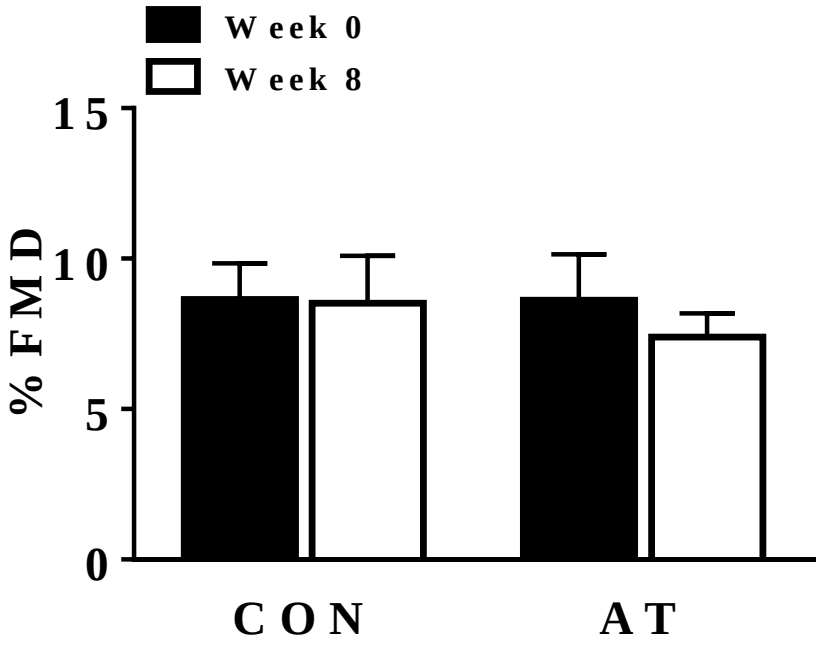

Figure 10 Brachial artery flow mediated dilation in CON and AT participants.

Resting FMD was unaffected from week zero to week eight in both CON and AT participants. Data presented as mean ± SE. Figure used with permission from Journal of Hypertension: July 2016 –Volume 34 – Issue 7 – p 1309-1316. Wolters Kluwer Health Lippincott Williams & Wilkins©

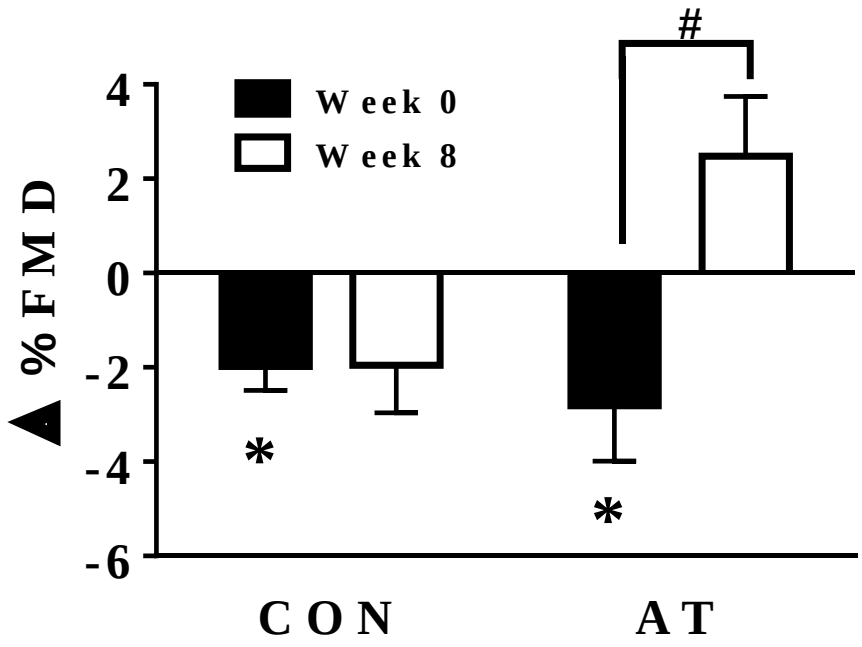

Figure 11 The effect of acute bilateral leg press on brachial artery flow mediated dilation in CON and AT participants.

Pre to post-acute bilateral leg press ΔFMD% was significantly reduced post BLP at week zero (*; P <0.05) and trended towards a reduction at week eight in CON (P=0.08). In AT participants pre to post-acute bilateral leg press ΔFMD% was significantly reduced at week zero (*; P < 0.05) but was preserved following eight weeks of training. At week eight AT participant's post BLP brachial artery ΔFMD % was positive and significantly different compared to week zero. (#; P <0.05). Data presented as mean ± SE. Figure used with permission from Journal of Hypertension: July 2016 – Volume 34 – Issue 7 – p 1309-1316. Wolters Kluwer Health Lippincott Williams & Wilkins©

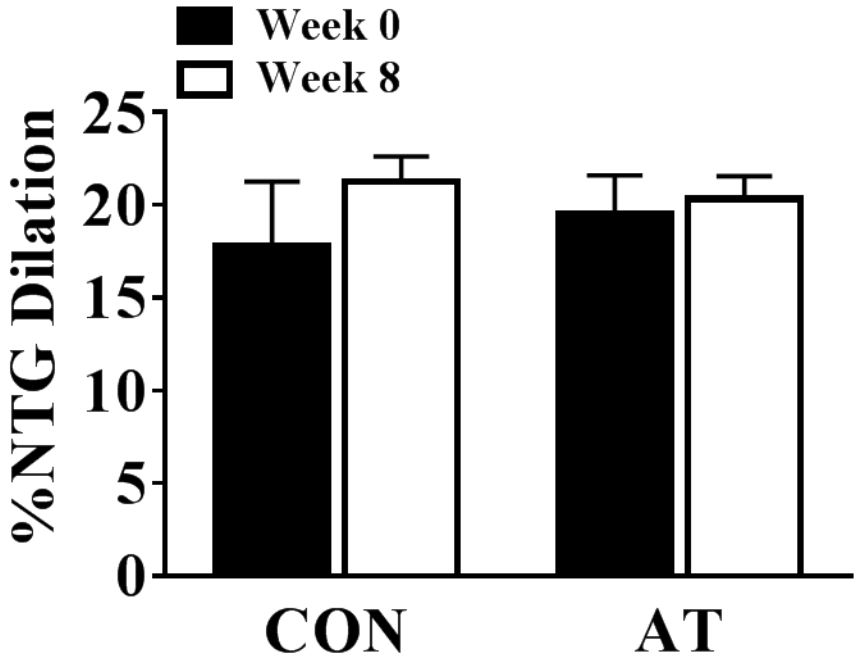

Figure 12 Nitroglycerin mediated dilation in CON and AT participants.

No significant between or within group differences in Nitroglycerin mediated dilation were observed. Data presented as mean ± SE. Figure used with permission from Journal of Hypertension: July 2016 –Volume 34 – Issue 7 – p 1309-1316. Wolters Kluwer Health Lippincott Williams & Wilkins©

Table 3 Brachial artery vascular characteristics of study participants across time points.

	CON (n=9)				AT (n=10)			
	Week 0		Week 8		Week 0		Week 8	
	Pre BLP	Post BLP	Pre BLP	Post BLP	Pre BLP	Post BLP	Pre BLP	Post BLP
Baseline diameter (mm)	3.5 ± 0.3	3.5 ± 0.3	3.4 ± 0.32	3.4 ± 0.3	3.6 ± 0.4	3.6 ± 0.4	3.7 ± 0.46*	3.7 ± 0.5*
Brachial artery FMD (%)	9.3 ± 4.2	7.4 ± 4.2	9.3 ± 4.1	6.9 ± 4.7	8.6 ± 4.8	5.7 ± 4.5	7.7 ± 2.79	10.6 ± 3.7*
Acute BLP Δ FMD (%)	-1.9 ± 1.4		-2.3 ± 2.7		-2.9 ± 0.4		2.9 ± 3.0*†	
Baseline BFV (cm/sec)	71.1 ± 5.7	73.4 ± 8.9	69.9 ± 6.9	72.9 ± 14.6	70.2 ± 13.4	73.0 ± 20.7	71.4 ± 8.6	74.8 ± 12.6
Peak BFV (cm/sec)	135.9 ± 17.9	130.0 ± 19.5	137.2 ± 16.2	142.9 ± 14.0	137.3 ± 20.9	131.3 ± 19.3	137.1 ± 17.2	147.9 ± 21.9
Baseline SR, s-1	208.5 ± 32.1	215.0 ± 35.8	204.9 ± 30.7	213.8 ± 47.5	201.8 ± 53.8	208.4 ± 67.3	197.9 ± 41.8	208.3 ± 60.6
Peak SR, s-1	362.7 ± 50.5	352.6 ± 52.3	366.0 ± 43.8	390.5 ± 39.4	363.9 ± 86.7	355.7 ± 78.8	352.2 ± 68.5	368.3 ± 78.7
Normalized FMD	0.026 ± 0.01	0.020 ± 0.01#	0.025 ± 0.01	0.018 ± 0.01#	0.3 ± 0.01	0.02 ± 0.1#	0.022 ± 0.01	0.0 ± 0.01
NTG dilation, %	20.3 ± 5.1		20.4 ± 3.8		19.8 ± 3.6		20.7 ± 2.7	

All values expressed as mean ± SD.*Statistical significance between groups after BLP (P <0.05). Within group statistical significance #acute BLP effect and †chronic training effect (P <0.05). CON = control, AT = aerobic training, Acute BLP Δ FMD, % = change in FMD post-acute leg press exercise vs. pre-acute leg press exercise, BFV = blood flow velocity, SR = shear rate. Table used with permission from Journal of Hypertension: July 2016 –Volume 34 – Issue 7 – p 1309-1316. Wolters Kluwer Health Lippincott Williams & Wilkins©

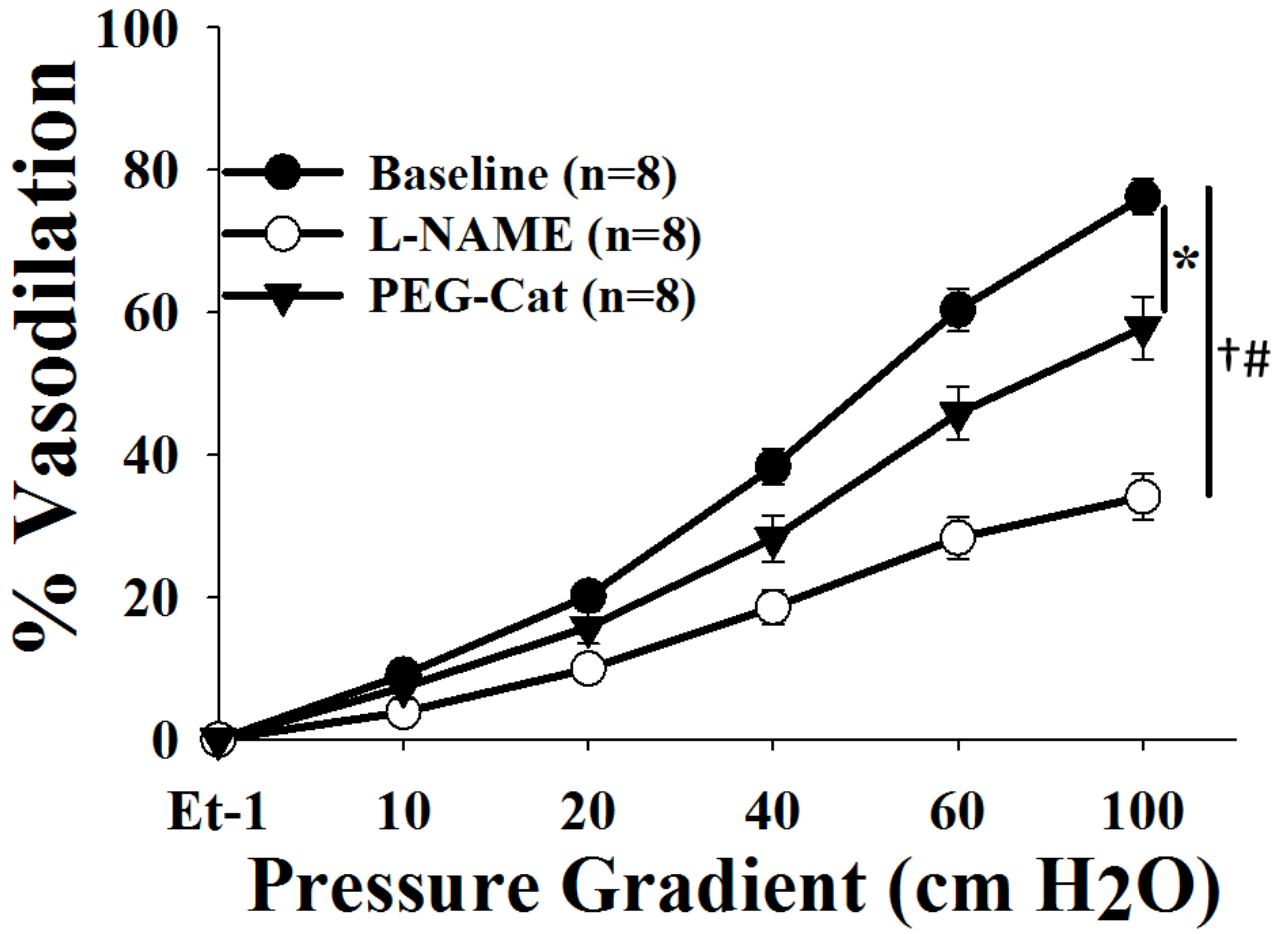

Figure 13 Resting vasodilation and vasodilatory phenotype at week seven in control participants.

Vasodilation was significantly impaired by H_2O_2 scavenging via PEG-Cat (*; P <0.05) and even more so by NOS inhibition via L-NAME. L-NAME reduced microvessel FMD at rest significantly compared to both the baseline condition (#) and the PEG-Cat pretreatment condition (†). (P <0.05) Data presented as Mean ± SE. Figure used with permission from Journal of Hypertension: July 2016 –Volume 34 – Issue 7 – p 1309-1316. Wolters Kluwer Health Lippincott Williams & Wilkins©

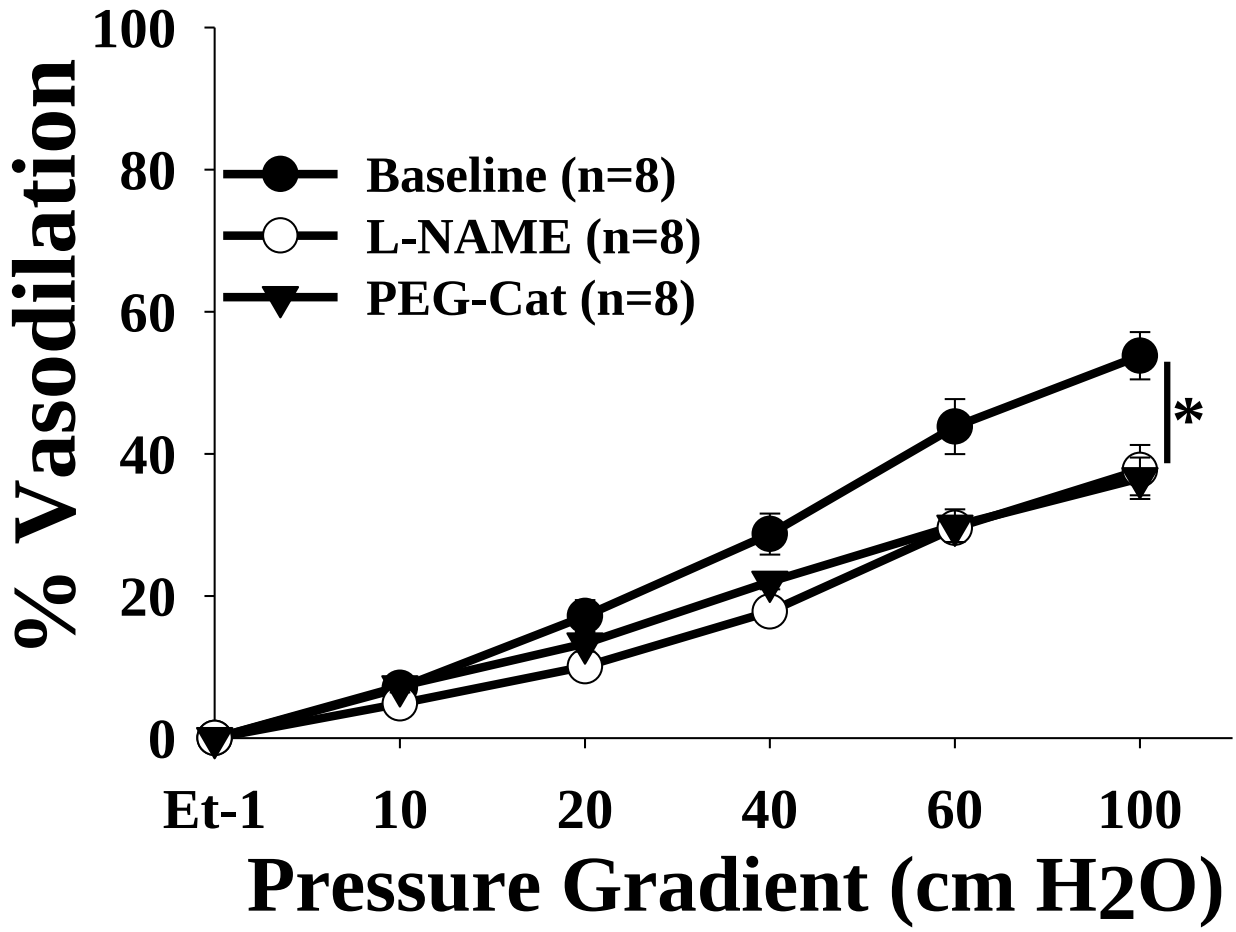

Figure 14 Post-acute bilateral leg press vasodilatory phenotype at week eight in control participants.

Both L-NAME and PEG-Cat further reduced an already attenuated microvessel FMD (*; P <0.05). The there was no difference between the reduction induced by L-NAME or PEG-Cat at 60 or 100 cmH_2O. Data presented as Mean ± SE. Figure used with permission from Journal of Hypertension: July 2016 –Volume 34 – Issue 7 – p 1309-1316. Wolters Kluwer Health Lippincott Williams & Wilkins©

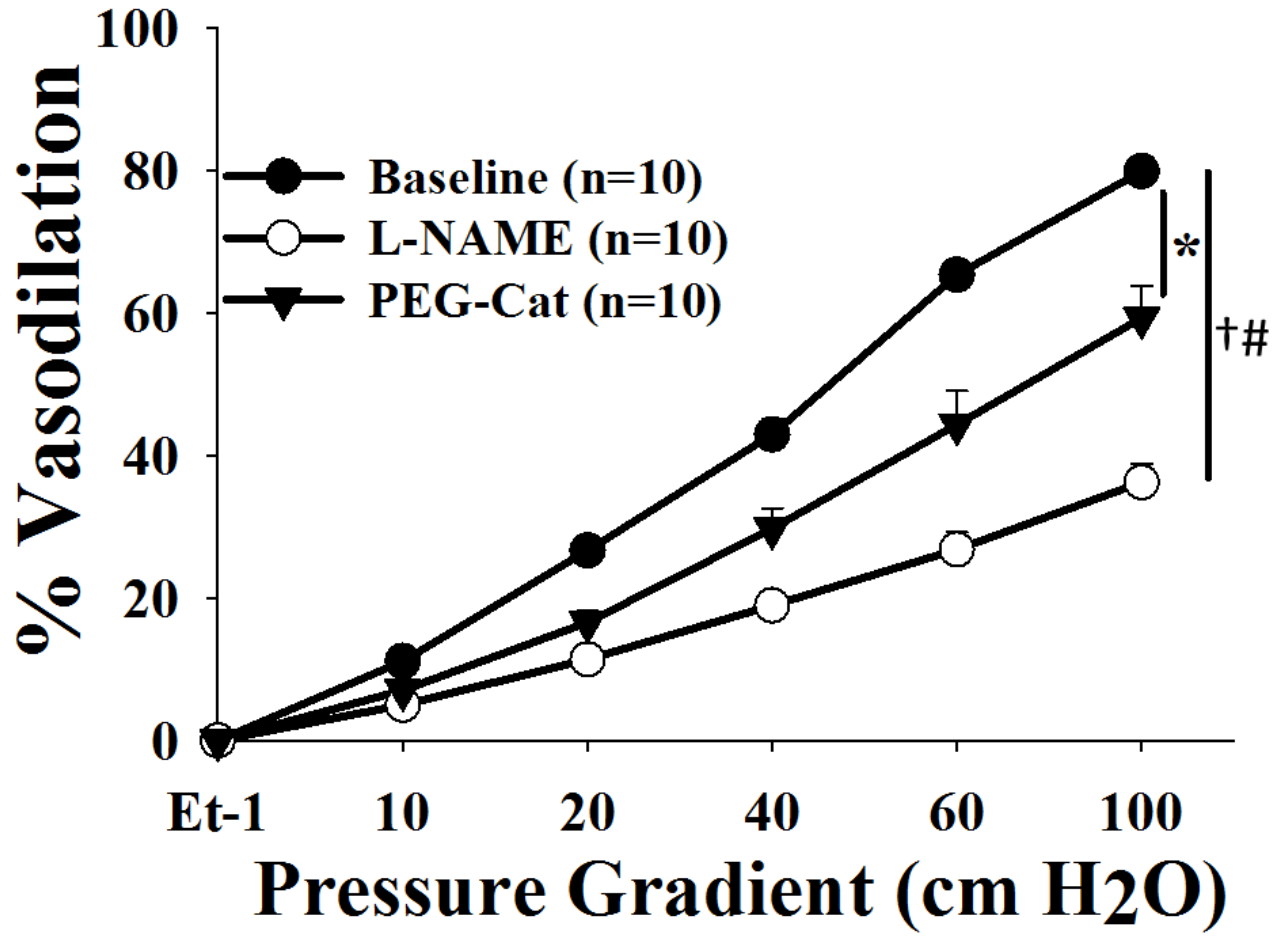

Figure 15 Resting vasodilatory phenotype at week seven in aerobic exercise trained participants.

Vasodilation was significantly impaired by H_2O_2 scavenging via PEG-Cat (*; P <0.05) and even more so by NOS inhibition via L-NAME. L-NAME reduced microvessel FMD at rest significantly compared to both the baseline condition (#) and the PEG-Cat pretreatment condition (†). (P <0.05) Data presented as Mean ± SE. Figure used with permission from Journal of Hypertension: July 2016 –Volume 34 – Issue 7 – p 1309-1316. Wolters Kluwer Health Lippincott Williams & Wilkins©

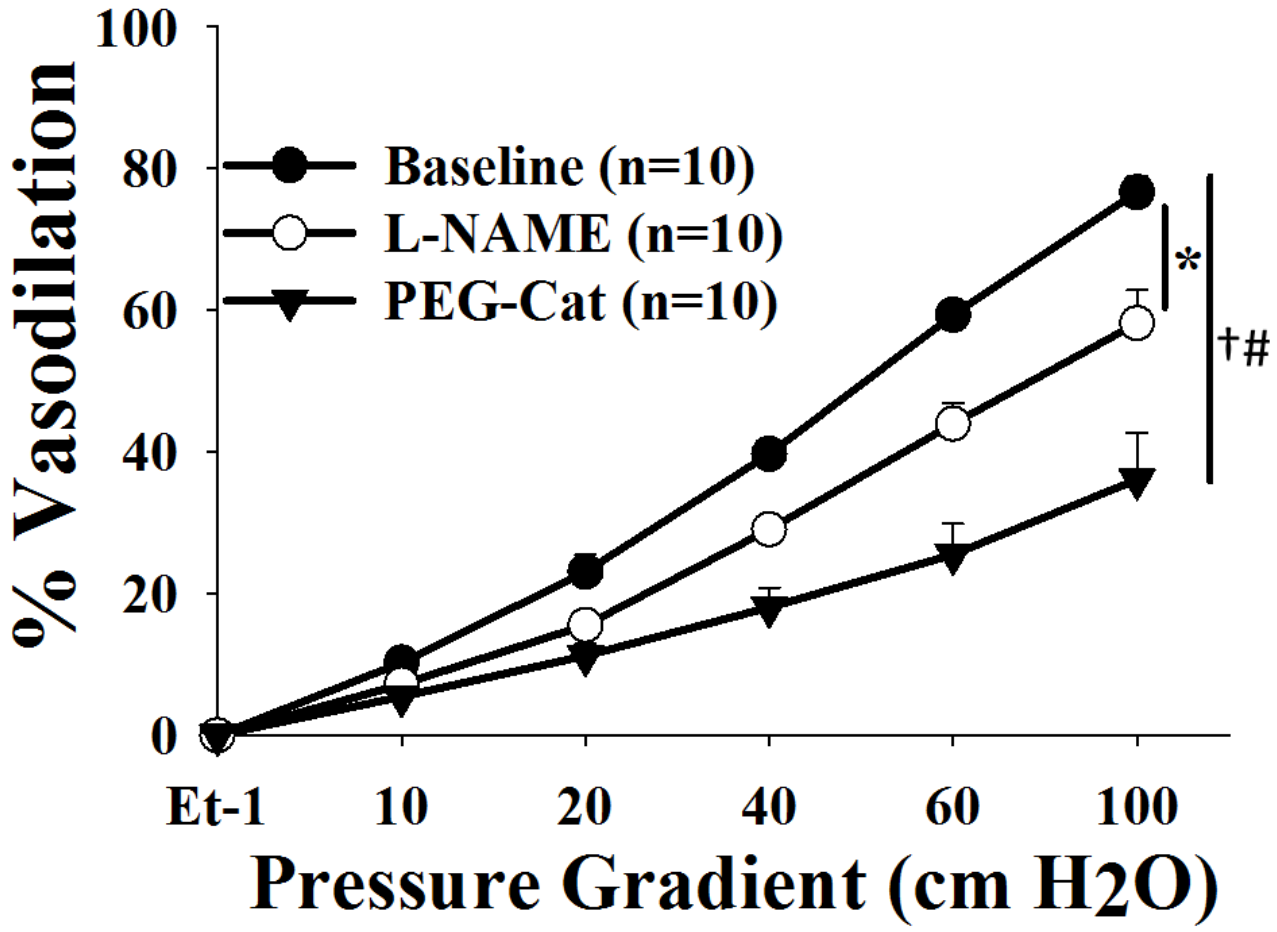

Figure 16 Post-acute bilateral leg press vasodilatory phenotype at week eight in exercise trained participants.

Microvessel FMD following an acute bout of bilateral leg press at week eight was preserved in aerobic exercise trained participants. In contrast to the resting condition, post-acute bilateral press microvessel FMD was significantly impaired by NOS inhibition with L-NAME (*; P <0.05) but more so by H_2O_2 scavenging with catalase. PEG-Cat significantly reduced microvessel FMD following acute bilateral leg press compared to both baseline (†) and with L-NAME (#). (P <0.05) Data presented as Mean ± SE. Figure used with permission from Journal of Hypertension: July 2016 –Volume 34 – Issue 7 – p 1309-1316. Wolters Kluwer Health Lippincott Williams & Wilkins©

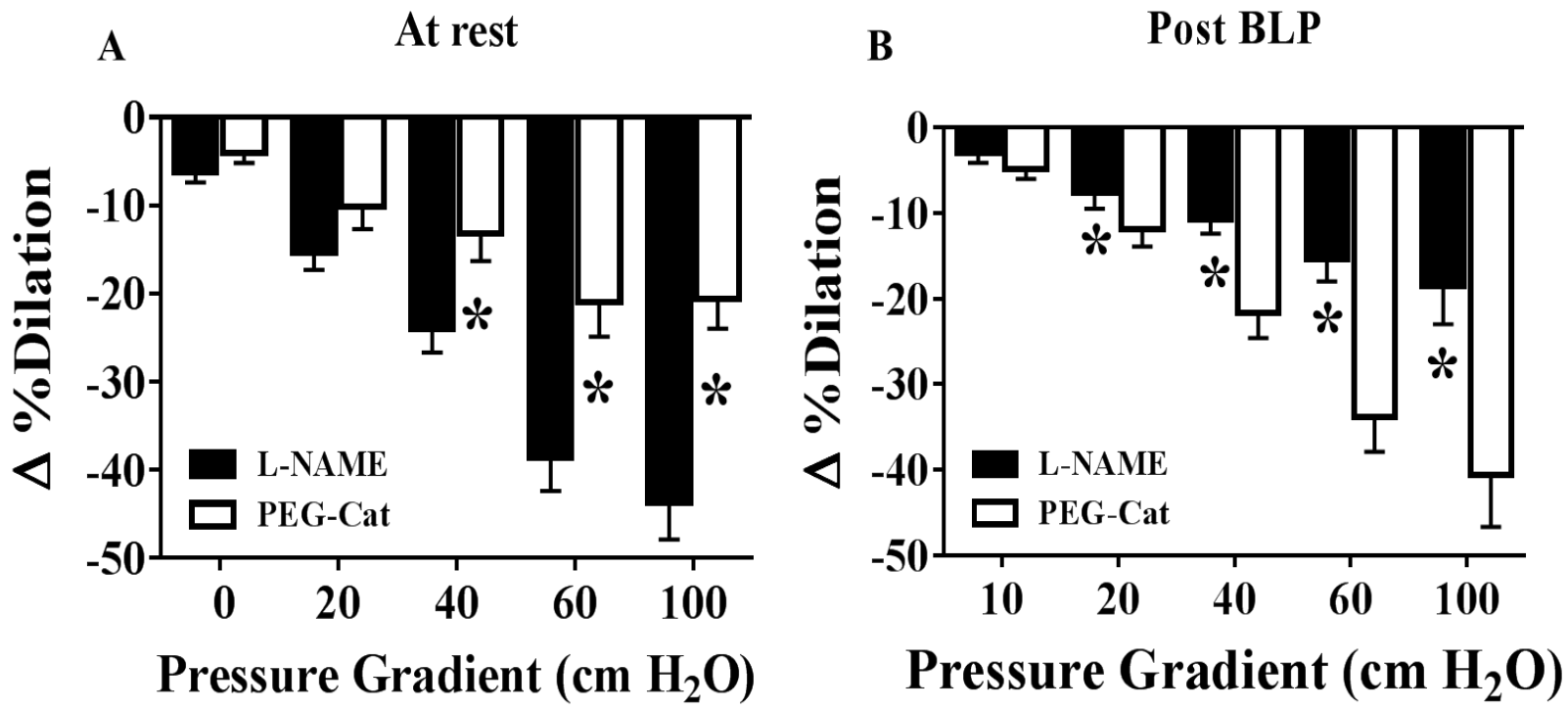

Figure 17 Post-acute bilateral leg press elicited a phenotypic switch in microvessels obtained from AT participants.

These data are summarized responses of the inhibitory effects of NOS inhibition and H_2O_2 scavenging at each pressure gradient.(A) In the resting condition L-NAME reduced microvessel FMD significantly more so than PEG-Cat at Δ 40, 60, and 100 cmH_2O. (B) Following an acute bout of bilateral leg press PEG-Cat reduced microvessel FMD significantly more so than L-NAME at Δ 20, 40, 60, and 100 cmH_2O. These findings indicate a switch from NO mediated dilation at rest to H_2O_2 mediated dilation post-acute bilateral leg press. (n= 10 in each condition; * P <0.05) Data presented as Mean ± SE. Original representation of the data. No permissions needed.

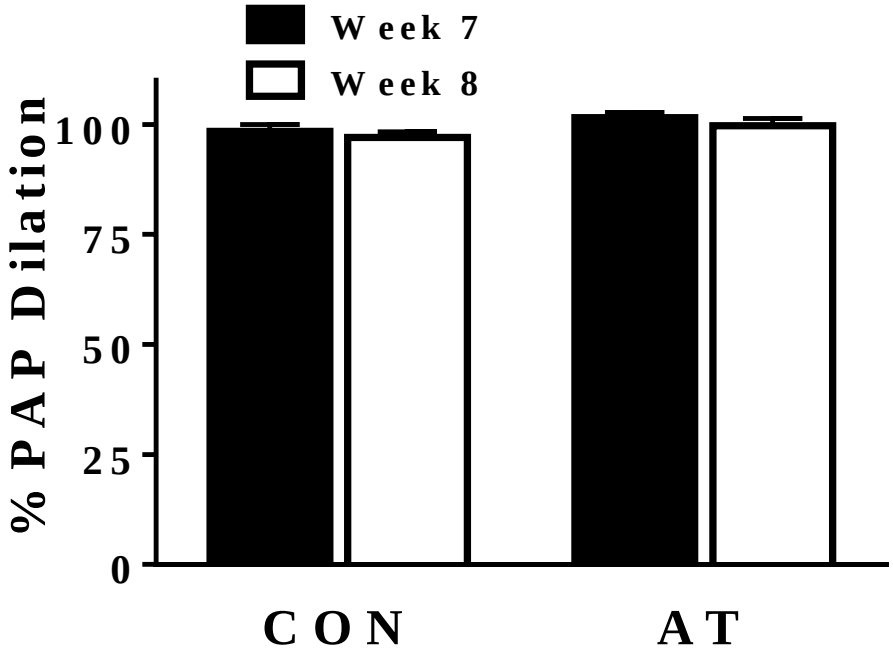

Figure 18 Papaverine mediated dilation in microvessels obtained from CON and AT participants.

No significant differences in Papaverine mediated dilation were observed between (CON vs AT) or within groups (Week 7 vs Week 8). Data presented as mean ± SE. Original representation of the data. No permissions needed.

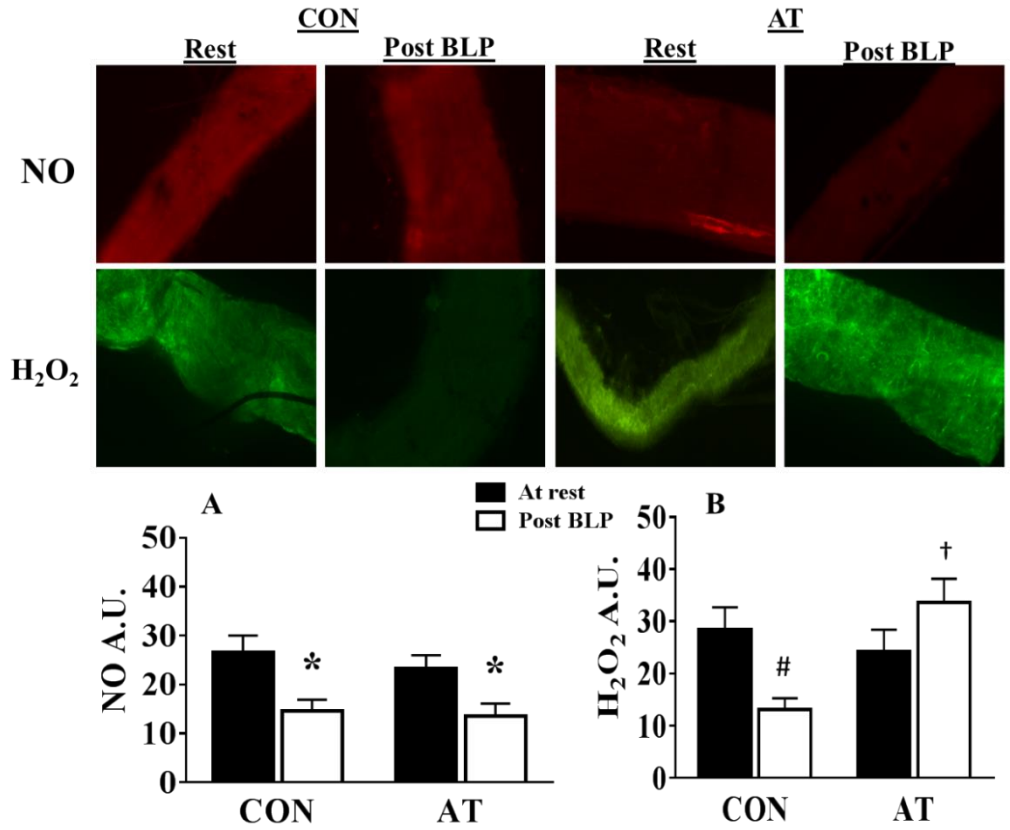

Figure 19 NO. and H2O2 fluorescence pre and post-acute bilateral leg press.

(A) Nitric oxide production was decreased following an acute bout of bilateral leg press compared to at rest in both CON (n=5 pre BLP; n=4 post BLP) and AT participants (n=6 pre BLP; n=6 post BLP). (*; P <0.05) **(B)** Hydrogen peroxide production was decreased following acute bilateral leg press compared to at rest in CON (n=7 pre BLP; n=4 post BLP) participants (#; P <0.05) but elevated in AT participants (n=8 pre BLP; n=8 post BLP). (†; P <0.05) Data are presented as mean ± SE. Figure used with permission from Journal of Hypertension: July 2016 –Volume 34 – Issue 7 – p 1309-1316. Wolters Kluwer Health Lippincott Williams & Wilkins©

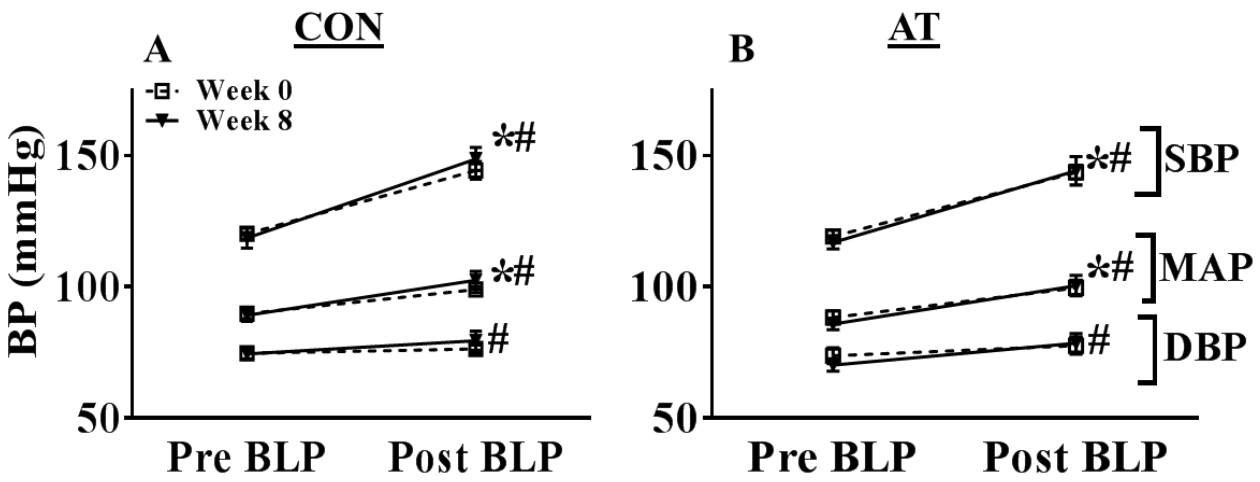

Figure 20 Pre – post bilateral leg press changes in blood pressure.

Resting blood pressure and blood pressure responses to acute bilateral leg press were similar in control (A) and aerobically trained (B) participants. At both week zero (*) and week eight (#) the acute bout of bilateral leg press induced significant elevations in systolic blood pressure and consequently mean arterial in both control and aerobically trained participants. (P <0.05) Diastolic blood pressure was elevated following acute bilateral leg press only at week eight in both control and aerobically trained participants .(P <0.05) SBP = systolic blood pressure, MAP = mean arterial pressure, DBP = diastolic blood pressure. Data are presented as mean ± SE. Figure used with permission from Journal of Hypertension: July 2016 –Volume 34 – Issue 7 – p 1309-1316. Wolters Kluwer Health Lippincott Williams & Wilkins©

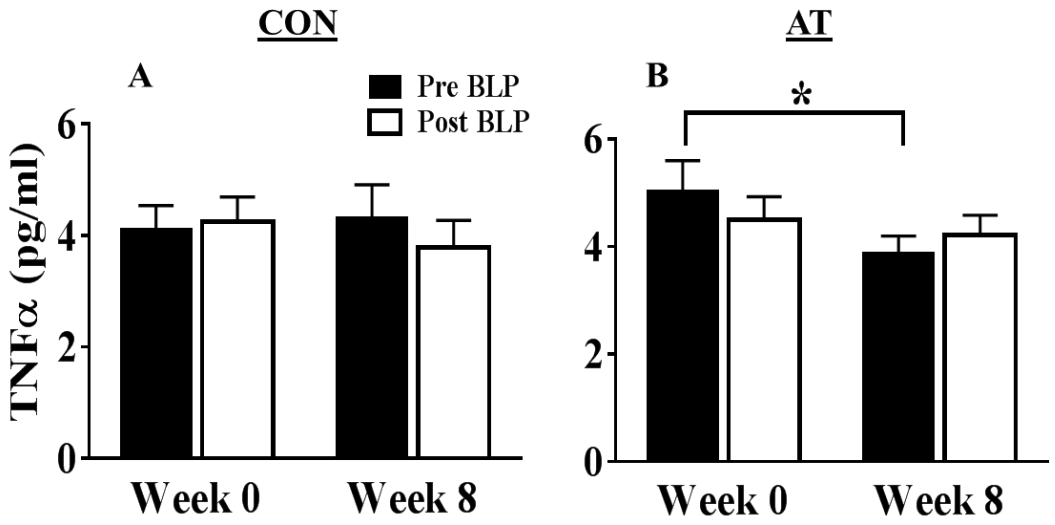

Figure 21 Pre – post bilateral leg press changes in plasma TNF-α.

(A) Plasma TNF-α was not changed by acute bilateral leg press in control participants and resting levels did not change over the course of the study intervention in control participants. **(B)** Plasma TNF-α was not changed by acute bilateral leg press in aerobically trained participants but resting levels were reduced over the course of the study intervention. (P <0.05) Data are presented as mean ± SE. Figure used with permission from Journal of Hypertension: July 2016 –Volume 34 – Issue 7 – p 1309-1316. Wolters Kluwer Health Lippincott Williams & Wilkins©

III. D Discussion

The key findings of this study are that in previously sedentary, overweight and obese individuals regular aerobic exercise training prevented acute physical exertion-induced brachial artery and microvessel dysfunction; and (*2)* preserved microvessel vasodilator function following acute exertion is H_2O_2 dependent following aerobic exercise training in obese and overweight adults. Brachial artery FMD was significantly reduced following acute resistance exercise at week zero in both AT and CON groups. This is not of surprise as participants in both groups entered the study sedentary and overweight or obese. Brachial artery FMD and microvessel FMD were maintained at week eight in AT participants. In contrast brachial artery and microvessel FMD were reduced following acute resistance exercise at week eight in CON participants. The preserved dilation in microvessels following acute resistance exercise in AT participants at week eight was blocked by scavenging of H_2O_2 via catalase. In addition, microvessels obtained from aerobic exercise trained participants exhibited a concomitant increase in H_2O_2 production after acute leg press exercise. Another supporting surrogate measure of H_2O_2 production at the systemic level was increased plasma superoxide dismutase in AT participants. Taken together these findings suggest that H_2O_2 production in exercise trained overweight and obese individuals is increased following acute strenuous exertion and acts as the principle mediator of microvessel vasodilation. These findings are agreeance with previous vascua sectional data from our group in exercise trained normal weight individuals [36].

The H_2O_2 mediated microvessel dilation in aerobic exercise trained participants following acute BLP was in contrast to resting microvessel FMD which was $NO^{\cdot}$ mediated in both AT and CON participants. This suggests that H_2O_2 can act as an alternative vasodilator under conditions in which $NO^{\cdot}$ bioavailability is reduced. This is similar to previous studies in the coronary and peripheral circulations. [39, 40, 292]

Previous findings from our group indicate that microvessels from lean exercise conditioned adults exposed to high intraluminal pressure are protected from arterial dysfunction via H_2O_2 mediated microvessel [28, 36, 37]. Animal models have also found regular aerobic exercise restore arterial function in the microcirculation in an H_2O_2 - dependent fashion. In a porcine model of coronary artery disease, regular exercise restored microvessel dilatory function via H_2O_2 mediated dilation [174]

Our participants did not experience a change in resting FMD which is agreeance with previous findings in exercise studies of this duration [293, 294]. Based on previous findings from our group indicating that high pressure exposure in isolated microvessels mimics the effects of acute strenuous exertion, our data supports the hypothesis that high pressure is the predominant factor contributing to 1) reduced blood vessel dilatory function in sedentary individuals and 2) the observed acute phenotypic switch from $NO^{\cdot}$ mediated dilation to H_2O_2 mediated dilation in isolated microvessels. The current study was not designed to specifically test the acute hypertension response to exercise and vascular dysfunction in our participants. However we did observe that plasma TNF-α was unchanged which suggests that the vascular dysfunction was not attributable to a global inflammatory response. It should be noted that had we tested more markers of inflammation (e.g. interleukin-6) our results may have been different. Harris et al [27] found an increase in circulating IL-6 in plasma collected immediately after exercise and no change in TNFα. In agreeance our group found no difference in TNFα or C-reactive protein (CRP). Thus IL-6 appears to be the most sensitive marker to assess in order to determine if acute bouts of exercise evoke an increase in inflammation which may play a role in endothelial cell responses [295, 296]. The reduction in resting TNF-α following eight weeks of aerobic exercise suggests participants were exposed to less chronic inflammation which may also play a small role. Lastly another major

contributing factor to favorable post-acute resistance exercise arterial function in our aerobic exercise trained participants could have been dampened sympathetic outflow in response to the acute bout of leg press compared to their sedentary counterparts. [261, 297]

This study has several limitations. Biopsies were not obtained at baseline (week 0) due to research volunteer burden of 4 biopsies in a single protocol. Instead we recruited an age and BMI-matched control group to compare microvessel FMD at week seven and following bilateral leg press exercise at the end of the intervention. Essentially this means we made a cross sectional comparison between an exercise trained cohort and a sedentary cohort. In addition, the resting biopsies and the post-acute exercise biopsies at week seven and eight were obtained one week apart which means female participants were exposed to varying menstrual hormone status at these two visits. The size of human microvascular tissue biopsies is comparatively small and thus we could not measure protein expression in our sample. Additionally, we did not perform dilatory dose responses to H_2O_2. Therefore, we could not determine whether aerobic exercise conferred a greater dilatory responsiveness to H_2O_2 itself. Third, our design allowed mechanistic insight into preserved microvessel function, but the $NO^{\cdot}$ or H_2O_2 contributions to brachial artery FMD in the pre- versus post- acute resistance exercise conditions was not investigated. As we already performed biopsies and this was an intervention study, intra-arterial infusion would have only complicated the design further. Fourth, we were not able to control for menstrual status. While we acknowledge this is an important factor, it was not feasible while scheduling around the availability of the practitioner to perform biopsies, and the scheduled exercise training sessions. Fifth, we did not measure beat to beat blood pressure and used the auscultatory method Thus, we underestimated the peak blood pressure responses. Lastly, the invasive nature of the gluteal biopsies in addition to the eight-week training intervention necessitated our limited sample size. With a final sample size

of ten AT and nine CON subjects, our study was not powered to test sex or race differences. However, we did still find significance in our primary outcome measures. In addition, the findings of this study align with previous studies of similar size and using a similar study population from our lab [27-29]. Nonetheless the relationship between regular exercise and the preservation of vascular function in response to various stressors shown to induce vasodilator dysfunction is an area that needs further exploration, particularly in the microcirculation.

This study provides evidence that regular aerobic exercise is associated with improvements in conduit artery and microvessel FMD following acute strenuous exercise in overweight and obese individuals and that H_2O_2 mediated dilation is the mechanism responsible for preserved microvessel vasodilator function following acute exertion. While our study used acute strenuous physical exertion as a stressor, a number of studies demonstrate that higher fitness may protect against numerous acute stressors which may evoke vascular dysfunction including mental stress [260], second hand smoke [298], excess alcohol consumption [287], high sugar [299], and high fat meals. [300, 301] These sources of acute stress to the vascular endothelium are ubiquitous and many of them are linked to higher incidence of cardiovascular disease [3]. Further research into the mechanisms through which regular exercise protects the vasculature from these assaults is needed. In conclusion regular aerobic exercise protects against acute exertion induced vascular dysfunction in previously sedentary, overweight and obese individuals and involves an H_2O_2 dependent mechanism.

Chapter IV Aerobic Exercise Dampens Acute High Intraluminal Pressure Induced Oxidative Stress in the Microvasculature

IV. A Introduction

Hypertension is the number one risk factor for cardiovascular disease (CVD), the number one cause of death in the United States. [302] Endothelium dysfunction has also been shown to provide prognostic value for predicting CVD [59, 303]. Hypertension and endothelium dysfunction are intertwined, however the temporal relationship between the two has yet to be fully elucidated. [49, 304, 305]

Our group has primarily focused on the role of high pressure inducing vascular dysfunction using an isolated vessel approach to expose resistance arteries to increased intraluminal pressure. [36, 41, 157] Hypertension is associated with excessive oxidative stress (i.e. increased superoxide (O_2^-) production) in the endothelium with the two primary sources being NADPH Oxidase (NOX II) and the mitochondria with the local renin angiotensin system (RAS) playing a role [41, 187, 244, 306]. In isolated adipose resistance arteries blockade of the local RAS via the AT_1R blocker Losartan and the ACE inhibitor Captopril prevents high pressure (150 mmHg) induced impairment of acetylcholine induced dilation and increased O_2^- production [41]. In isolated carotid arteries exposure to elevated intraluminal pressure has been shown to cause impaired acetylcholine induced dilation, greater NOX II activity, and excessive production of O_2^-. Carotid arteries transfected with siRNA to inhibit NOX II activity displayed improved acetylcholine-induced vasodilation and produced less O_2^- following high intraluminal pressure exposure [244].

A recent interesting finding is that isolated microvessels from exercise trained individuals have dampened O_2^- in response to high intraluminal pressure compared to microvessels from sedentary individuals and microvessels from exercise trained individuals are protected from high pressure induced vasodilator dysfunction [35, 244]. Furthermore hydrogen peroxide (H_2O_2) appears to be the mediator of preserved vasodilation in adipose resistance arteries from exercised trained individuals. It is well understood that O_2^- reduces $NO^{\cdot}$ bioavailability and that it can be converted to H_2O_2 via dismutation. However, the source of H_2O_2 in these resistance arteries was not established in previous studies. Exercise increases SOD in the aortic endothelium [173] and exogenous SOD has been shown to rescue resistance artery FMD following high intraluminal pressure [307]. Thus, an exercise induced increase in SOD expression would seem to be a likely mechanism for reduced O_2^- and improved H_2O_2 mediated vasodilator function in response to high intraluminal pressure in microvessels obtained from exercise trained individuals. However, this has yet to be elucidated

Therefore, the purpose of this study was to determine if regular exercise protects against high intraluminal pressure induced vascular dysfunction via greater expression SOD isoforms in adipose resistance arteries obtained from control and exercised mice. In order to determine the effect of exercise and shear on NADPH oxidase, we measured NOX II subunits in order to confirm NOX II is the predominant source of O_2^- following exposure to high intraluminal pressure in resistance arteries down stream of local RAS. In order to strengthen our findings, we also used a cell model to investigate the effects of Ang II on NOX II activity, O_2^- production, and the ability of increased laminar shear stress to dampen these effects.

We hypothesized that exercise would preserve vascular function following exposure to high intraluminal pressure and that this could be attributed to an increase in SOD and H_2O_2 mediated dilation along with a concomitant decrease in NOX II. We also hypothesized our cell model would implicate shear stress as the primary mediator of exercise related adaptations in regards to NOX II and SOD.

IV. B Methods

Exercise Protocol

Twelve to sixteen week old male C57BL/6J mice (obtained from Jackson Laboratory, Bar Harbor, ME USA) were used for this study in collaboration with Dr. Tohru Fukai [UIC College of Medicine]. Mice ran on an exercise wheel (12.7 cm diameter) placed inside their cage for two weeks. On average the mice ran ~ six km per 24 hours in line with previous studies. [308, 309] Mice were initially acclimatized to the exercise wheels for 48 hours to ensure that they would run. After this period, mice were given permanent access to the wheels. Mice ran on the wheels during the night (2000h to 0800h) as they are nocturnal. While a locked wheel may have provided control mice environmental enrichment, a single mouse was housed in each cage with no wheel. Mice were fed normal mouse chow *ad libitum*. Within two hours of their last exercise bout, mice were anesthetized with isoflurane and sacrificed for experimentation. The animals were not fasted at the time of sacrifice. Animal experiments were conducted in accordance with UIC'S Animal Care and Use Committee.

Resistance artery flow mediated dilation

Resistance artery flow mediated dilation experiments were performed as previously described. [35, 288] Bilateral hind limb subcutaneous fat pads were removed from mice and placed in cold (4^oC) HEPEs buffer (140mM NaCl, 4mM KCl, 1.2mM $MgCl_2$, 5mM glucose, 10mM

HEPES, and 1.2mM $CaCl_2$). Following dissection of vessel arcades, single resistance arteries were cannulated on glass micropipettes (internal diameters of ~30 to 50 µm) in an organ chamber filled with Krebs solution (123mM NaCl, 4.7mM KCl, 1.2mM $MgSO_4$, 2.5mM $CaCl_2$, 16mM $NaHCO_3$, 26µM EDTA, 11mM glucose, and 1.2mM KH_2PO_4). Remaining arteries were snap frozen in liquid nitrogen for molecular biology experiments described below. Both ends of the cannulated resistance arteries were secured on the micropipettes with surgical ties (10-0 nylon Ethilon monofilament sutures). The organ chamber was then set up on the stage of an inverted microscope attached to a video camera, video monitor, and a video-measuring device (model VIA-100; Boeckeler) and continuously perfused at 40 ml/min (MasterFlex pump, Cole Parmer) with warm Krebs buffer. The organ chamber was aerated with a gas mixture of 21% O_2, 5% CO_2 and maintained at 37°C via a perfusion system with tubing connected to a thermostat (PC200, Thermo Scientific, NC, USA). The vessel was maintained at an intraluminal pressure of 60 cmH_2O for 30 min.

After intraluminal pressure of 60 cmH_2O was maintained for 30 minutes, vessels were constricted 40–60% with ET-1 (125 ± 5 pmol for CON mice and 115 ± 3 pmol for EX mice; N.S. Mean ± SE). Pre-constriction allows for greater observation of dilatory responses. Resistance artery FMD was produced by generating pressure gradients of Δ10, Δ20, Δ 40, Δ60, and Δ100 cmH_2O using Krebs-filled reservoirs and diameters were taken after three minutes. For the high pressure model, high Intraluminal pressure (HILP; 150 cmH_2O) was administered for 45 minutes followed by 15 minutes of re-equilibration at 60 cmH_2O, similar to the previous protocol published by our group [36, 41, 157]. Resistance artery FMD was measured in the absence and presence of the NOS inhibitor L^{NG}-Nitroarginine Methyl Ester (L-NAME; 100µM), H_2O_2 scavenger Polyethylene glycol-Catalase (PEG-Catalase; 500U/ml), AT_1r blocker Losartan (50µM), NOX II

inhibitors VAS2870 (2μM) and NSC23766 (10 μM), and SOD inhibitor, Zinc-diethyldithiocarbamate Zn-DDC (1mM), which were added in randomized order to the external bathing solution of the organ chamber either 30 minutes before pre-constriction with endothelin-1 (ET-1) or co-incubated during exposure before exposure to HILP. Maximal diameter of every vessel (endothelium-independent vasodilation) was determined in the presence of Papaverine (100 μM) at the end of each round of FMD. Microvessel FMD was calculated as the percent change from the ET-1 induced pre-constricted diameter relative to the maximal diameter measured at rest prior to ET-1 constriction. All chemicals other than VAS2870 and NSC23766 were obtained from Sigma-Aldrich Corporation (St. Louis, MissourI, USA). VAS2870 was obtained from Enzo life Sciences (East Farmingdale, New York, USA). NSC23766 was obtained from Tocris Bioscience (Bristol, United Kingdom).

Resistance Artery Angiotensin II dose responses

Resistance Artery vasoconstriction in response to Angiotensin II dose responses was performed as previously described [310]. Resistance arteries maintained at an intraluminal pressure of 60 cmH_2O were exposed to incremental doses of angiotensin II (10^{-10} to 10^{-6}M) for three minutes each to ensure that a steady state diameter had been achieved. Following the dose response vessels were maximally dilated using Papaverine (100 μM) to ensure the integrity of the smooth muscle cells ability to relax.

Resistance artery fluorescence

Superoxide and hydrogen peroxide production were measured in measured following cannulation and maintenance of 37 °C in a 20 ml aerated organ chambers bathed in Krebs solution chamber at an equilibration pressure of 60 cmH_2O for 30 minutes or following exposure to the

HILP protocol described above. Superoxide production was determined using 5μM Dihydroethidium (DHE). H_2O_2 production was assessed using 1 μM DCF-DA (2',7'-Dichlorodihydrofluorescein diacetate). Both dyes were purchased from Thermo Fisher Scientific (previously Invitrogen). After incubation with each dye simultaneously, vessels were exposed to flow via a pressure gradient of Δ60 cmH_2O created by changing the height of the Krebs reservoirs for 30 minutes. Prepared vessels were then excised from the glass micropipettes and subsequently mounted on slides with DAKO fluorescent mounting medium (Dako North America, Inc Carpinteria, CA, USA). Mounted vessels were then immediately examined via fluorescent microscopy (Eclipse 80i; Nikon, Japan). Acquired images were analyzed for fluorescence intensity in arbitrary units (AU) using NIH Image J software. All vessels fluorescence was measured three times along different lengths of the vessel. Background intensities were then subtracted and fluorescence was normalized to vessel size to account for auto-fluorescence. [290, 291]

Human Adipose Microvascular Endothelial Cells (HAMECs)

Experiments were performed on isolated Human Adipose Microvascular Endothelial Cells (HAMECs) obtained from ScienCell (Cedro Carlsbad, CA, USA). HAMECs were grown in supplier recommended endothelial cell growth medium supplemented with 5% fetal bovine serum, EC growth supplement, 100 U/ml penicillin, and 100 μg/ml streptomycin (ScienCell) at 37°C in a humidified incubator (5% CO_2).

Shear Stress Experiments

For shear stress experiments, HAMECs were grown to 80–90% confluence. Laminar shear stress was generated using a modified cone and plate design (Dr. Micahel Brown's laboratory) as previously described [181, 311, 312]. The cone used in these studies is designed to fit into a 20 x

100-mm tissue culture dish and contains an upper cap which rests on the top of the culture dish, thus minimizing evaporation and supporting the free rotation of the cone axle secured by ball bearings and a setscrew. Magnetism to rotate the cone is provided by magnets within a modified Thermo Scientific Super-Nuova multi-place stirrer allowing for up to four plates to undergo shear at a time (Thermo Fisher Scientific; Waltham, MA, USA). Rotation of the cone forces the fluid between the cone and plate (endothelial cell medium) to flow congruously with the rotation of the cone, thus producing a fluid shear stress on the cells located on the stationary plate. The angle between the cone and pate is negligible (~ 0.5^{o}) thus facilitating a uniform flow pattern over all of the specimen on the plate. For the high shear stress condition 20 dynes/cm^2 for 24 hours, a value used in several exercise mimetic models [23, 186, 189]. All shear experiments were conducted under sterile conditions. Passages five through eight were used for all experiments involving shear stress.

The use of a cone and plate to shear endothelial cell monolayers dates back to 1981 when Davies and colleagues first described a cone-plate apparatus used to generate uniform fluid shear stress on bovine aortic endothelial cells. The endothelial cell monolayer undergoes a change in cell shape from polygonal (a “cobblestone” appearance) to a uniformly oriented fusiform shape with flow [313, 314].

Cellular fluorescence

HAMECs were grown to ~90% confluence on cover slips pretreated for 24 hours with 2% bovine plasma fibronectin in basal media. Once the desired confluency was met, cells were incubated with the O_2^- indicator DHE (5μM), H_2O_2 indicator DCF-DA (1 μM) and co-treated with or without Losartan (50μM), NOX II inhibitors VAS2870 (2μM) and NSC23766 (10 μM), or the SOD mimetic Tiron (100 mM) for 30 minutes. For the final 15 minutes of incubation Angiotensin

II (400 nM) was added and for the final 10 minutes the nucleic acid stain Hoechst 33342 (1 μM) was added. Cells were then washed twice using cold PBS and mounted on slides with DAKO fluorescent mounting medium. Mounted slips were then examined via fluorescent microscopy (Eclipse 80i; Nikon, Japan). Acquired images were analyzed for fluorescence intensity in arbitrary units (AU) using NIH Image J software. At least 30 individual cells were analyzed and randomizer.org was used to generate random sequences of 10 cells to be chosen from each treatment of each passage. This ensured that cases from no one passage disproportionately weighed the data.

Sample preparation & Western Blot Determination of Protein Expression

Following dissection of microvessels from adipose tissue (excluding vessels used for resistance artery FMD experiments) all remaining vessels were homogenized and sonicated (Qsonica Q55 Sonicator Ultrasonic Processor; Cole Palmer) in 60 μL radiommunoprecipitation assay (RIPA) buffer (150 mM Nacl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50mM Tris, pH 8.0)(Sigma) containing 3X protease and 3X phosphatase inhibitor cocktails (Thermo Fisher Scientific). The 3X protease inhibitor cocktail includes 3mM AEBSF, 2.4μM aprotinin, 150μM bestatin, 45μM E-64, 60μM leupeptin and 30 μM pepstatin A stabilized in dimethylsulfoxide (DMSO). Constituents of the 3X phosphatase inhibitor cocktail include a proprietary blend of sodium fluoride, sodium orthovanadate, sodium pyrophosphate and beta-glycerophosphate. Resistance artery homogenate was then centrifuged at 17,000 RCF at 4°C for 20 minutes. Protein determination of microvessel homogenate was determined using a bicinchoninic acid assay. Vessel lysates were heated at 95^{o}C for 5 minutes at a 2:1 with a 5% β-mercaptoethanol Laemmli sample buffer.

Following treatment with shear and/or various agonists and antagonists, cells were washed twice with cold PBS then lysed with 250 μL of RIPA buffer enhanced with 3x protease and phosphatase inhibitor cocktail (as described above) and were manually harvested and collected into 1.7 mL posi-click cryo tubes (Denville Scientific). HAMEC lysates were sonicated and then centrifuged at 17,000 RCF for 20 minutes at 4°C. Protein determination of HAMEC lysates was determined using a bicinchoninic acid assay (Pierce, Thermo Fisher Scientific). The remaining HAMEC supernatants were transferred and boiled in a 2:1 with a 5% β-mercaptoethanol Laemmli sample buffer (Bio-rad ; 277.8 mM Tris-HCl, pH 6.8, 4.4% LDS, 44.4% (w/v) glycerol, 0.02% bromophenol blue) at 95°C for 5 minutes.

Immunoblotting

For immunoblotting 20 μg of protein was loaded into each well of BioRad 10% gels. Samples were processed by SDS-PAGE (35 - 45 minutes depending on MW of target proteins at 200 volts) and transferred to Polyvinylidene fluoride (PVDF) membranes (0.22μm BioRad). The transfer conditions included the use of a wet transfer at 4°C. The time of the transfer varied from 70 minutes to 90 minutes depending on the molecular weight of the target protein. The transfer voltage was 90 to 110 volts, depending on the molecular weight of the target protein. The membranes were blocked with LiCor blocking buffer (Lincoln, NE) for one hour at room temperature. The membrane was incubated with primary antibodies for gp91^{phox} and p47^{phox}: 1:1000 (Cell Signaling Technologies), GAPDH 1:5000 (Cell Signaling Technologies), S303/304-phosphorylated p47^{phox}: 1:500 – 1:1000 (Thermo Fisher), Actin: 1:2000 (Thermo Fisher), SOD I: 1:3300 (R&D), SOD II and SOD III: 1:1000 (Santa Cruz) in LiCor blocking buffer with 0.2% Tween-20 added. Secondary antibodies (1:15,000) were incubated for two hours in LiCor blocking buffer with 0.2% Tween-20 and 0.01% SDS added. Between the primary and secondary antibody

incubation and after the secondary antibody incubation membranes were washed in TBST four times for five minutes per wash. Membranes were transferred to TBS following the last wash and imaged within one hour using a LiCor Odyssey scanner. Boxes were manually placed around each band of interest, which returned near-infrared fluorescent values of raw intensity with intra-lane background subtracted using Odyssey analytical software. This protocol was develop based off Licor recommendations and reviewing recent methods papers [315-317].

Statistics

All resistance artery FMD comparisons were computed using two-way repeated measures ANCOVA using vessel diameter as a co-variate, followed by pairwise comparisons using Bonferroni's adjustment. One Way ANOVA with Tukey's *post hoc* was used to compare HAMEC fluorescent data. Western blot and resistance artery fluorescence data were compared using t-tests. Alpha was set at <0.05. With power $(1 - \beta)$ set at 0.80 and α at <0.05 five samples were needed for each condition, assuming 20% differences in treatment conditions, standard deviation of 10%, and allowing for two pairwise comparisons. In some cases, the effect size was so large that only four samples in each condition were needed to satisfy power $(1 - \beta)$ set at 0.80.

IV. C Results

Resistance artery FMD and fluorescence

Basal resistance artery FMD was similar in vessels obtained from control and exercised mice (Fig. 22). Following exposure to high intraluminal pressure, resistance artery FMD was reduced in microvessels obtained from control mice adipose (Fig. 23; $P < 0.01$ @ 10, 20, 40, 60 and 100 cmH_2O). Resistance artery FMD was preserved following high intraluminal pressure in resistance arteries obtained from exercised mice (Fig. 24).

Impaired resistance artery FMD vessels obtained from control mice was restored with inhibition of RAS via Losartan (Fig. 25, $P<0.01$ at 10, 20, 40, 60 and 100 cmH_2O) and NOX II via VAS2870 (Fig. 26, at 10, 20, 40, 60 and 100 cmH_2O) and NSC23766 (Fig. 27, at 20, 40, 60 and 100 cmH_2O). Angiotensin II induced constriction dose responses indicated that resistance arteries obtained from control mice were more sensitive to Ang II than resistance arteries obtained from exercised mice (Fig. 28, $P<0.01$ at 10^{-6} to 10^{-8}M).

Resistance arteries obtained from control mice exposed to high pressure produced a significantly increased amount of O_2^- compared to vessels that were maintained at 60 cmH_2O (Fig. 29A, $P<0.01$). In contrast resistance arteries obtained from exercised mice exposed to high pressure did not produce a significantly greater amount of O_2^- compared to vessels that were maintained at 60 cmH_2O (Fig. 29B).

The preserved resistance artery FMD in vessels obtained from exercised mice after exposure to high intraluminal pressure was significantly reduced by PEG-CAT (Fig 30 $P<0.01$ at 10, 20, 40, 60 and 100 cmH_2O), L-NAME (Fig. 31 $P<0.01$ at 10, 40, 60 and 100 cmH_2O).The reduction via PEG-CAT was significantly greater than that of L-NAME (Fig. 34B). In contrast resistance artery FMD in vessels from exercise trained mice in the basal condition was inhibited by L-NAME (Fig 32 $P<0.01$ at 10, 20, 40, 60 and 100 cmH_2O) and PEG-CAT (Fig 33 $P<0.01$ at 60 and 100 cmH_2O) but the reduction via L-NAME was significantly greater than that of PEG-CAT (Fig. 34A). Further, resistance artery FMD in vessels obtained from exercised mice after exposure to high intraluminal pressure was significantly reduced by the endogenous Cu-Zn SOD inhibitor Zn-DDC (Fig 35 $P<0.01$ at 20, 60 and 100 cmH_2O) suggesting that SOD is the likely source of H_2O_2 following high pressure.

Resistance arteries obtained from exercise trained mice exposed to high pressure produced more H_2O_2 compared to vessels that were maintained at 60 cmH_2O (Fig. 36B, $P<0.01$). In contrast H_2O_2 generation in resistance arteries obtained from control mice exposed to high pressure was similar compared to vessels that were maintained at normal pressure (60 cmH_2O; Fig. 36A). Vasodilation to the $NO^{\cdot}$ donor Papaverine was similar between resistance arteries from control and exercise mice and there was no influence of high intraluminal pressure on the Papaverine response (Fig. 37).

HAMEC fluorescence

HAMECs exposed to Ang II produced a significantly more O_2^- compared to HAMECs maintained in the control condition and inhibition of RAS via Losartan or NOX II via VAS2870 or NSC23766 prevented increased O_2^- production. The exogenous SOD mimetic Tiron also prevented increased O_2^- production (Fig. 38, $P<0.01$).

Protein expression

Resistance arteries obtained from exercised animals expressed less of the membrane associated NOX II subunit $gp91^{phox}$ compared to control mice resistance arteries (Fig 39A, $P <0.05$). Resistance arteries obtained from exercised animals also expressed less of the cytosolic NOX II subunit $p47^{phox}$ compared to resistance arteries from control mice (Fig 39B, $P < 0.01$). In contrast, reistance arteries from exercised animals expressed greater amounts of cytoplasmic SOD (Fig. 40A SODII; $P < 0.01$), mitochondrial SOD (Fig. 40B SODII; $P < 0.01$), and extracellular SOD (Fig. 40C SOD III; $P<0.05$). In HAMECs exposed to high shear stress there was less expression of $p47^{phox}$and $gp91^{phox}$ than static cells (Fig. 41 A & B, $P < 0.01$ for both). In contrast

to the resistance arteries from exercise trained mice, high shear did not influence SOD I, SOD II, or SOD III expression (Fig. 42A-C). All blots were normalized to β-actin or GAPDH.

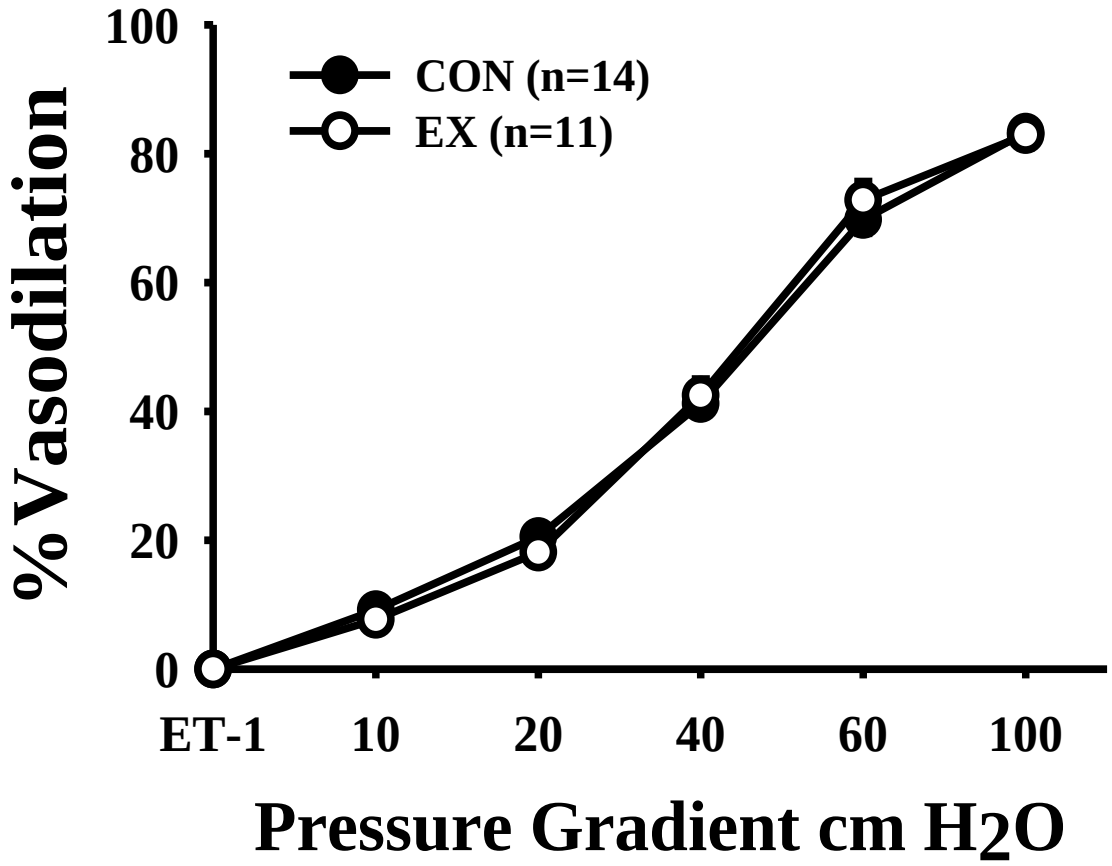

Figure 22 Resting resistance artery flow mediated dilation control and exercise mice.

There was no difference found in resistance artery flow mediated dilation between control (CON) and exercise mice (EX). Data are are presented as mean ± SE.

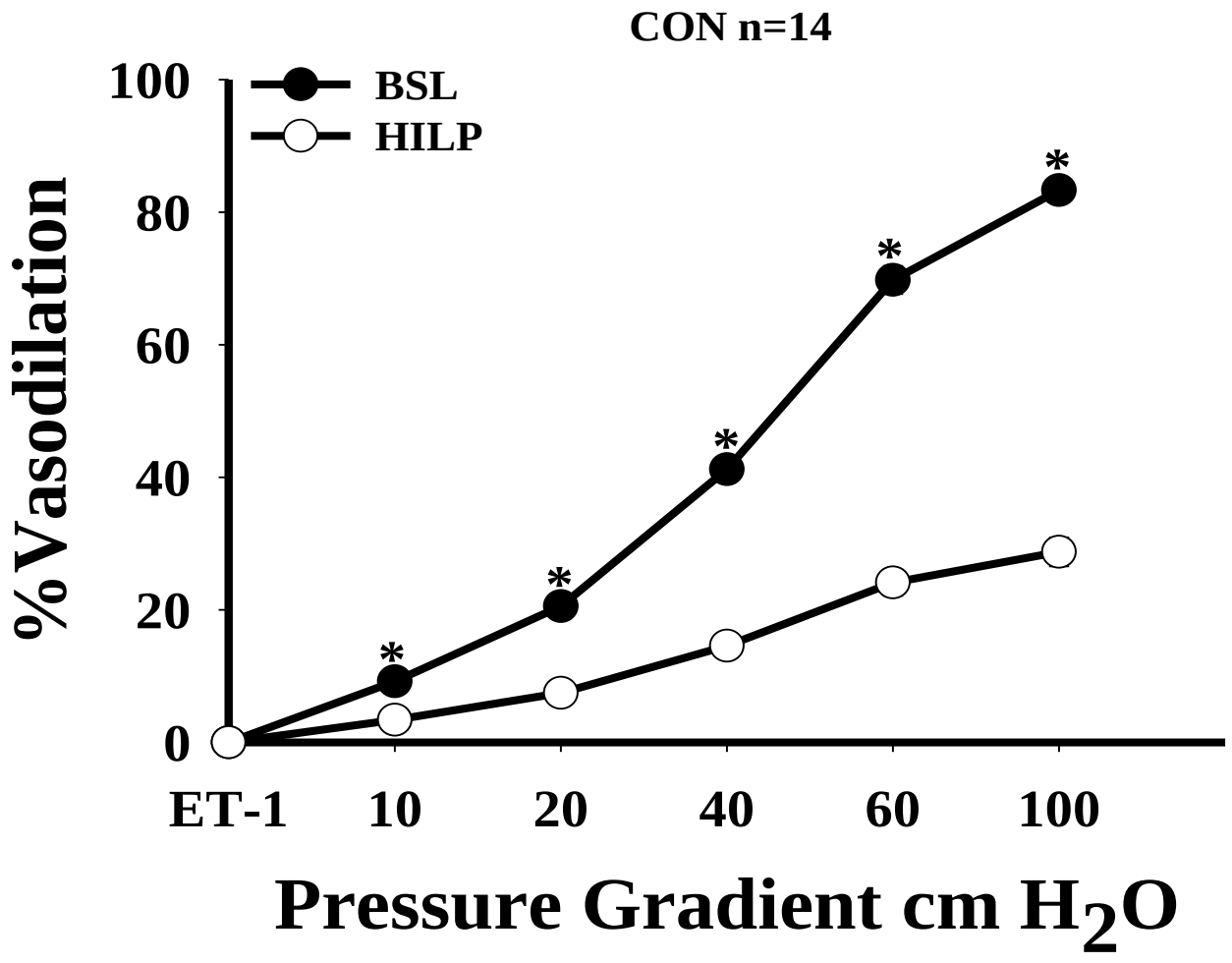

Figure 23 The effect of high intraluminal pressure on resistance artery flow mediated dilation in arteries from control mice.

High pressure (HILP) significantly reduced resistance artery FMD compared to baseline (BSL) in control mice arteries. *;*P* <0.01 at Δ 10, 20, 40, 60 and 100 cmH_2O. Data are presented as mean ± SE.

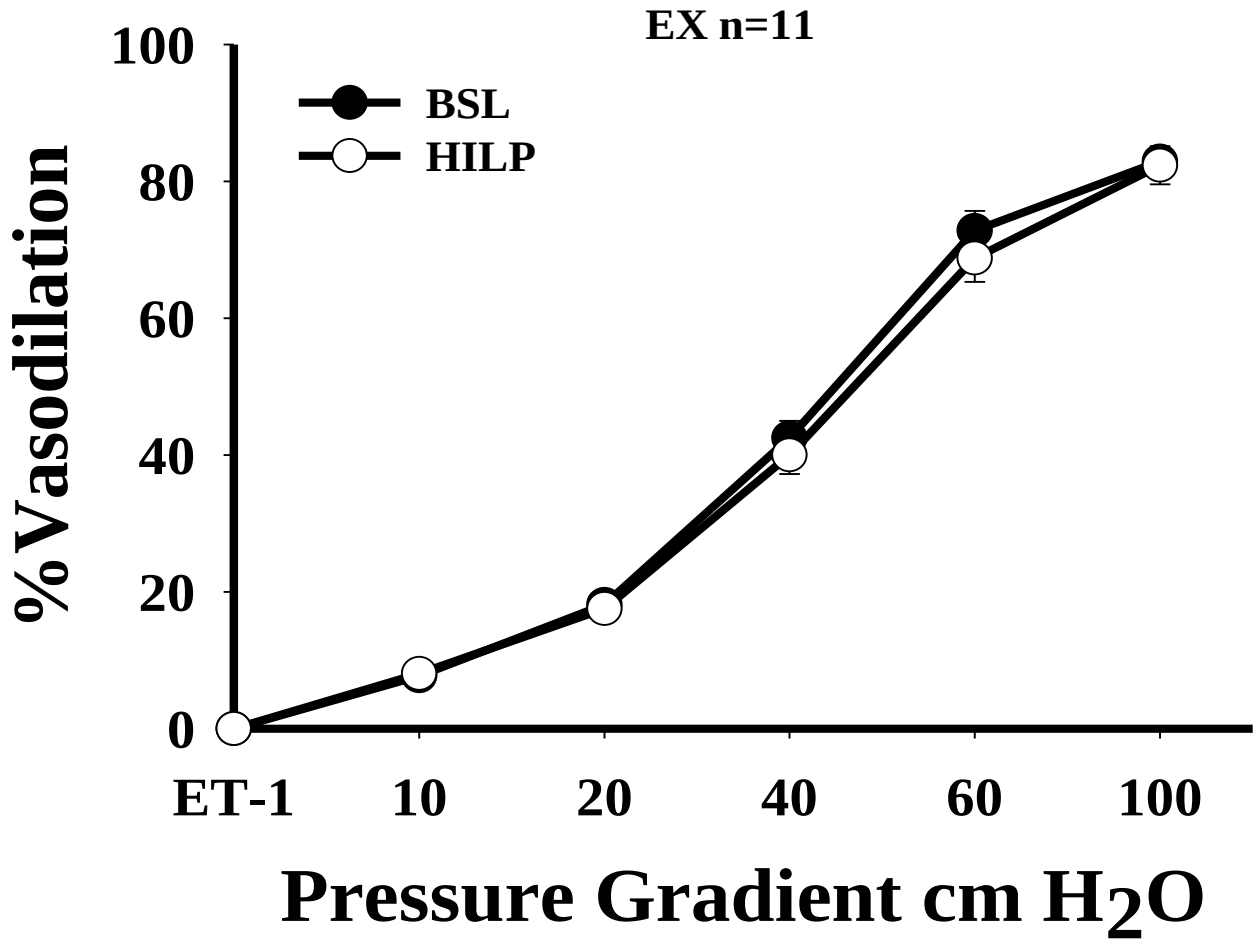

Figure 24 The effect of high intraluminal pressure on resistance artery flow mediated dilation in arteries from exercised mice.

There was no difference in resistance artery FMD following high pressure exposure (HILP) compared to baseline (BSL) in exercised mice arteries. Data are presented as mean ± SE.

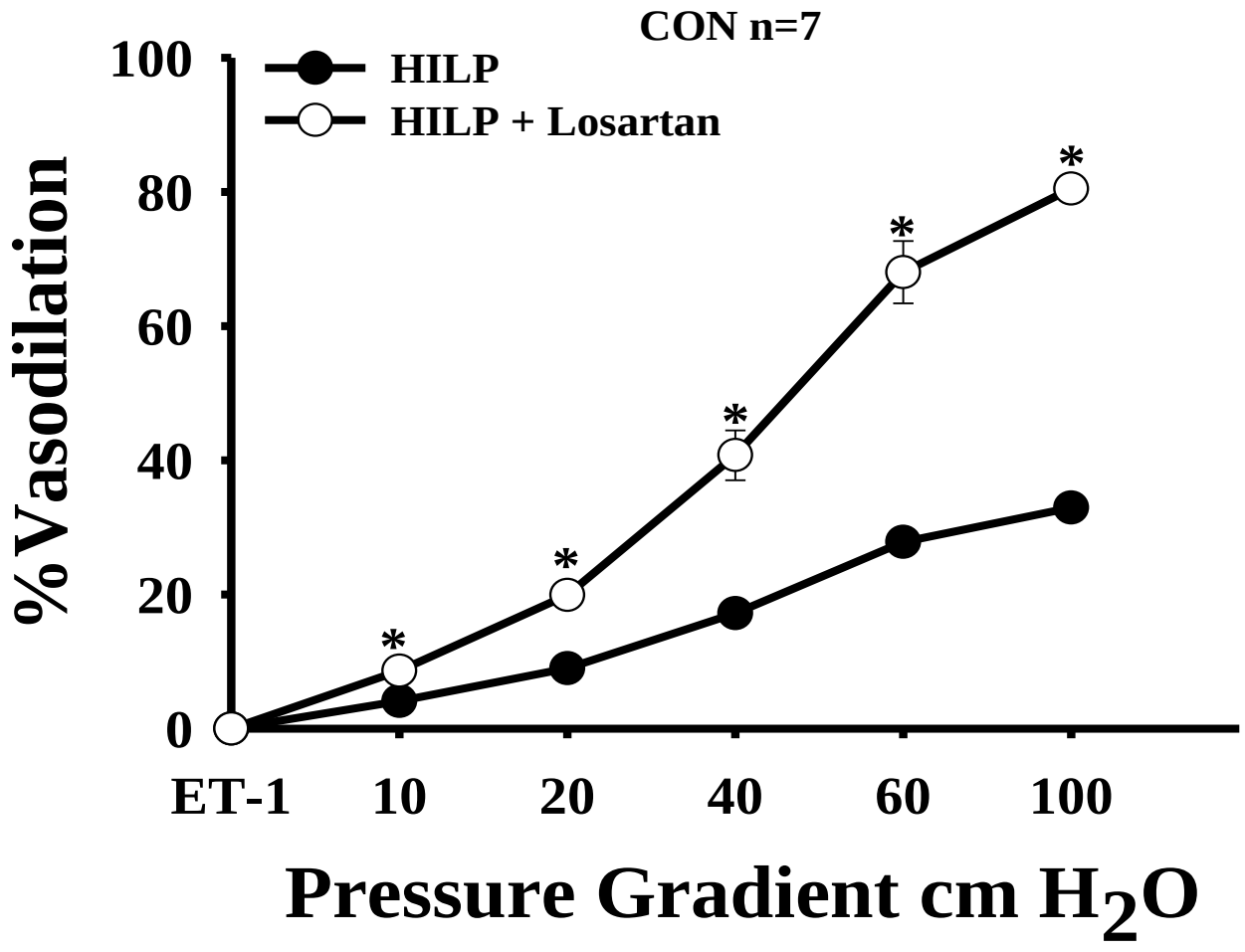

Figure 25 The effect of Angiotensin type I receptor blockade on high intraluminal pressure induced resistance artery vasodilator dysfunction in arteries from control mice.

Blockade of the AT_1r with Losartan (HILP + Losartan) restored high pressure (HILP) induced impairment in resistance artery FMD in vessels obtained from control mice. *;P <0.01 at Δ 10, 20, 40, 60 and 100 cmH_2O. Data are presented as mean ± SE.

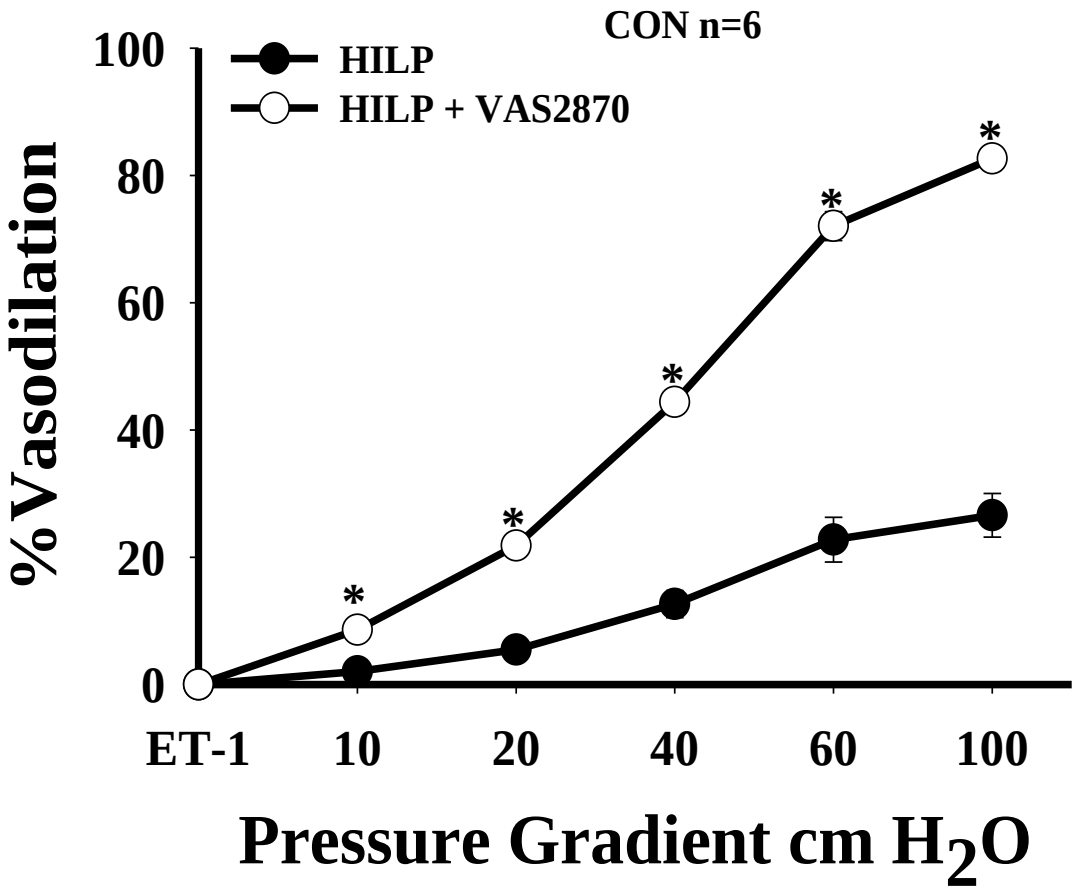

Figure 26 The effect of NADPH Oxidase II inhibition on high intraluminal pressure induced resistance artery vasodilator dysfunction in arteries from control mice.

Blockade of NOX II with VAS2870 (HILP + VAS2870) restored high pressure (HILP) induced impairment in resistance artery FMD in vessels obtained from control mice. *;*P* <0.01 at Δ 10, 20, 40, 60 and 100 cmH_2O. Data are presented as mean ± SE.

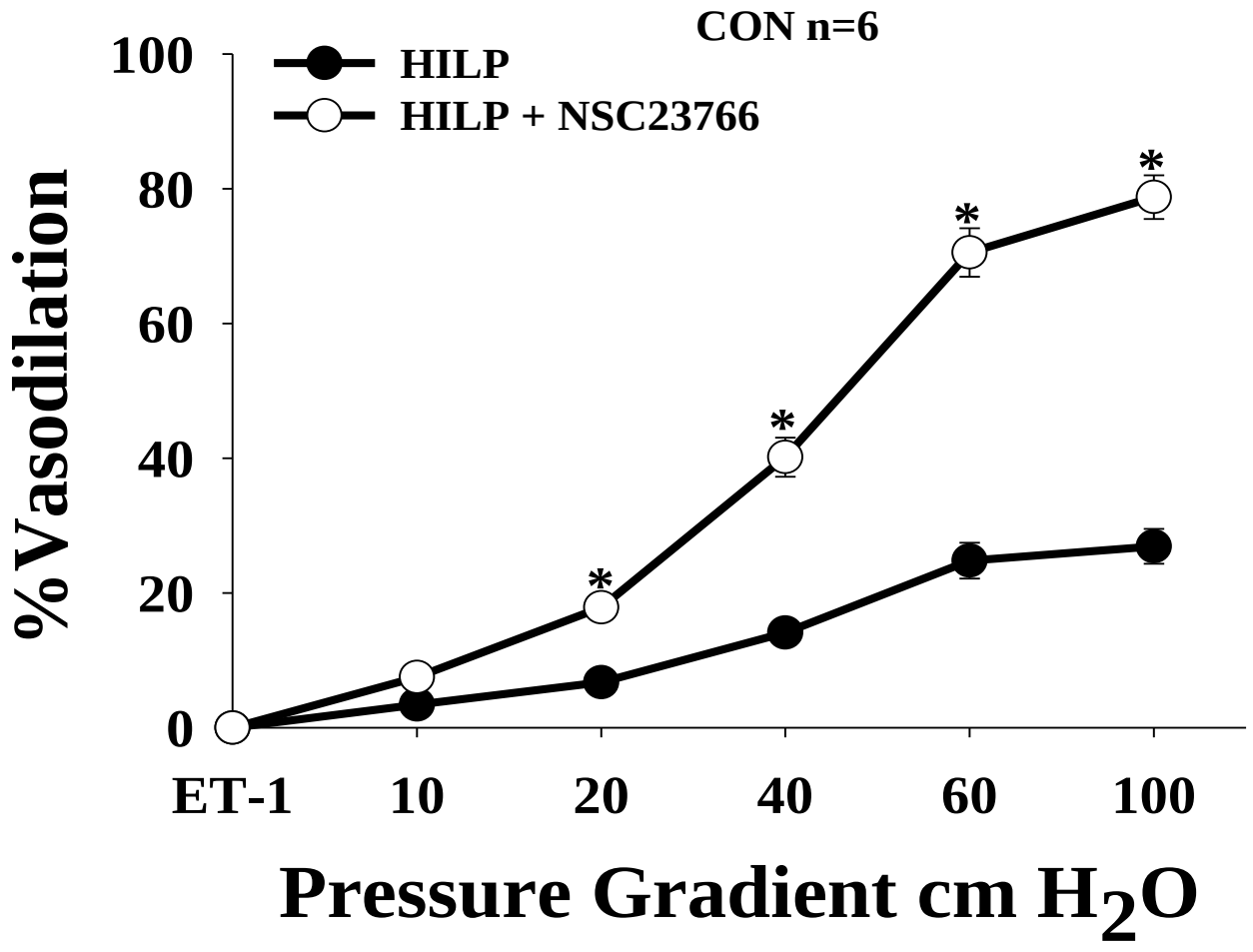

Figure 27 The effect of RAC/NOX II inhibition on high intraluminal pressure induced resistance artery vasodilator dysfunction in arteries from control mice.

Blockade of NOX II though inhibition of RAC1 GEF with NSC23766 (HILP +NSC23766) restored high pressure (HILP) induced impairment in resistance artery FMD in vessels obtained from control mice. *;P <0.01 at Δ 20, 40, 60 and 100 cmH_2O. Data are presented as mean ± SE.

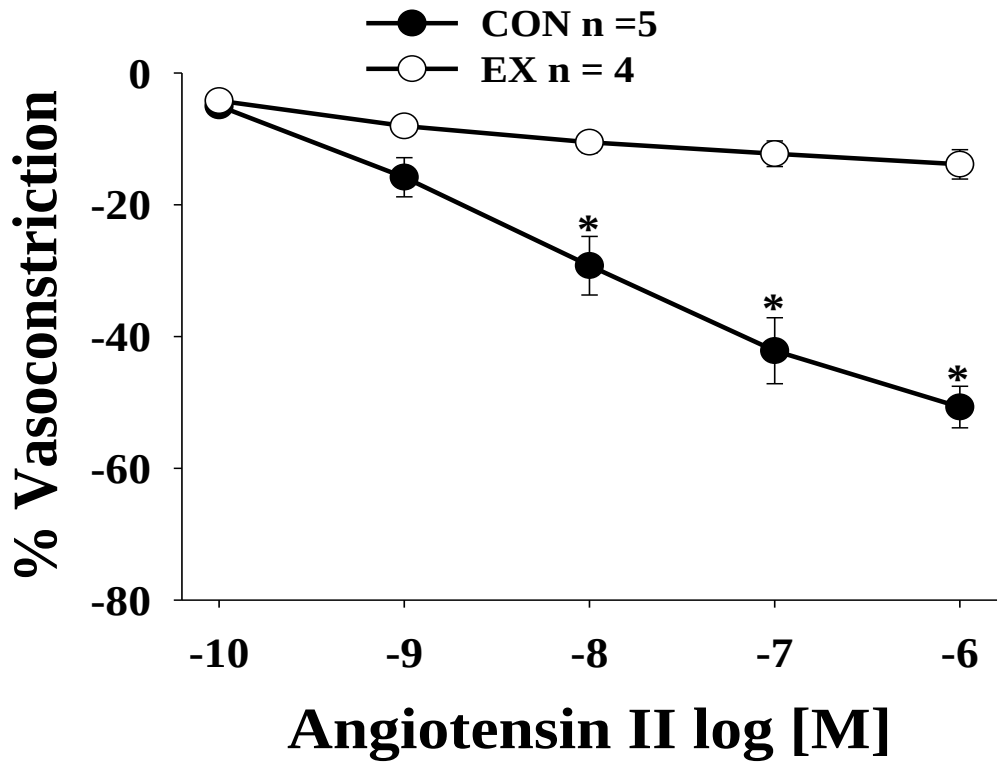

Figure 28 The effect of exercise on Angiotensin II-induced constriction.

Resistance arteries from exercised mice (EX) were significantly less sensitive to Ang II induced constriction compared to resistance arteries from control mice (CON). *;P <0.01 at 10^{-6} to 10^{-8}M. Data presented as mean ± SE.

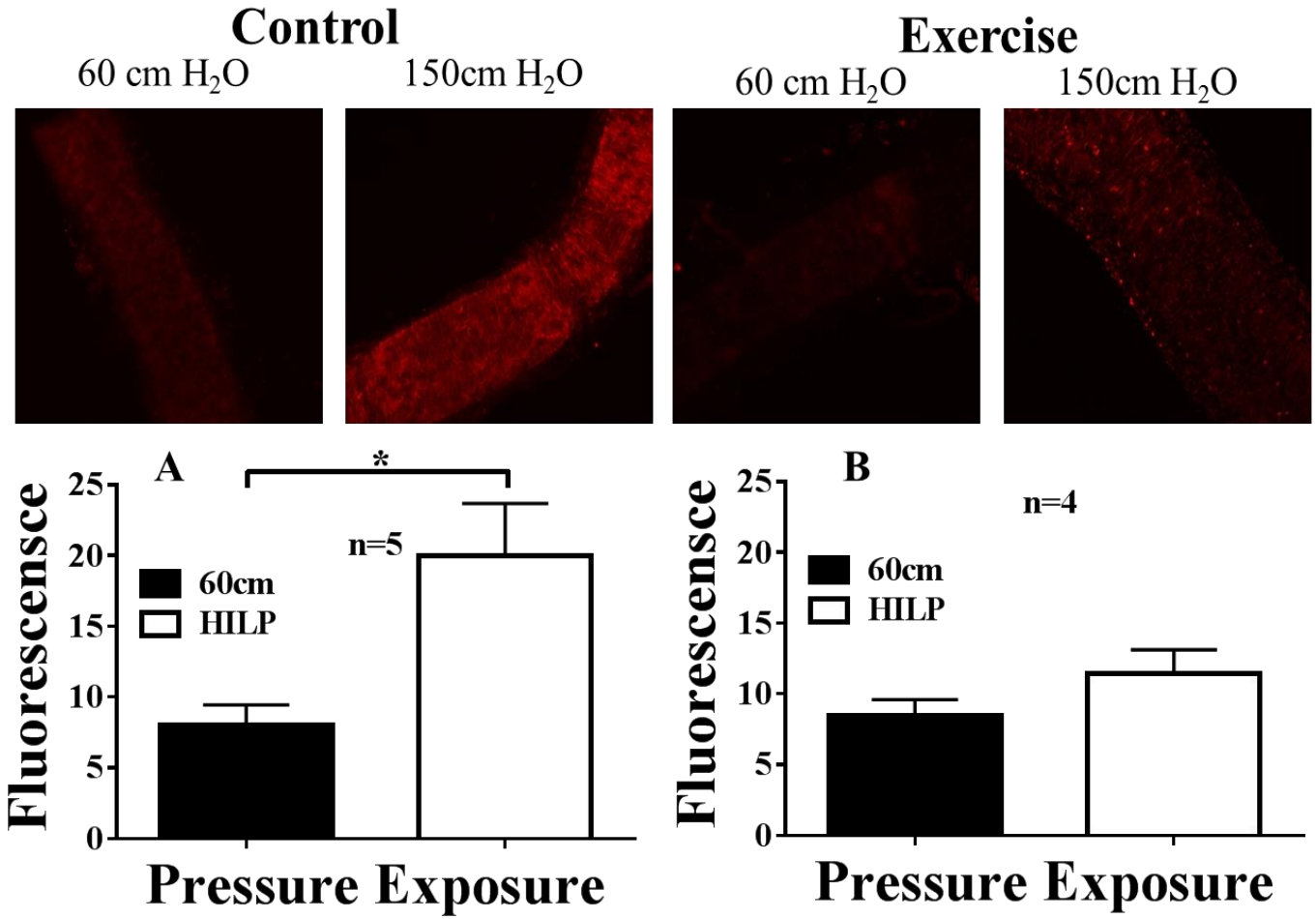

Figure 29 The influence of high intraluminal pressure on superoxide production.

(A) In arteries obtained from control mice high pressure exposure (HILP) evoked a significant increase in superoxide production compared to baseline (60 cmH_2O). *;*P* <0.05. **(B)** In arteries obtained from exercised mice high pressure exposure did not evoke a significant increase in superoxide production. Data presented as mean ± SE.

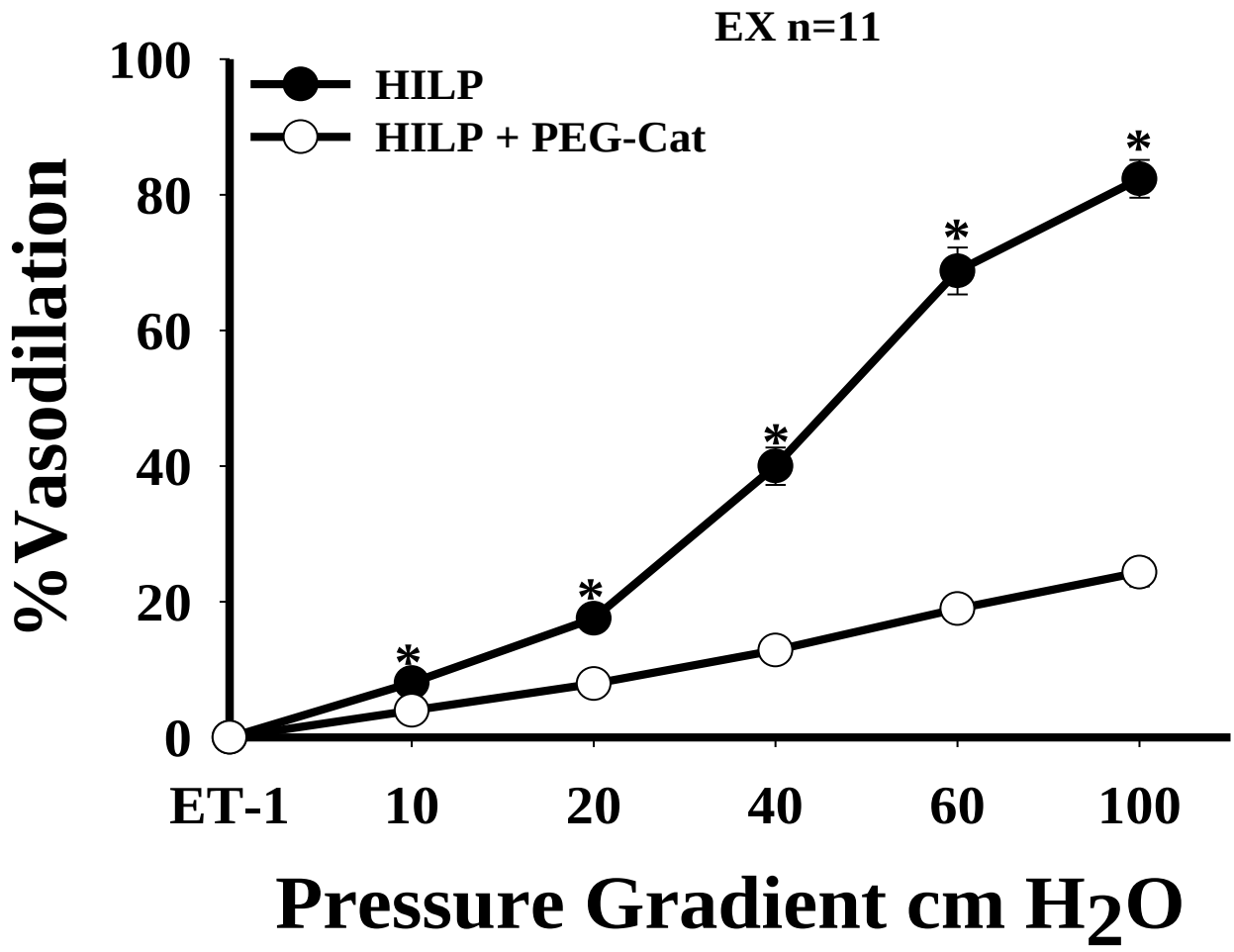

Figure 30 The effect of hydrogen peroxide scavenging on post high intraluminal pressure resistance artery vasodilator function in arteries from exercised mice.

With high pressure, scavenging of hydrogen peroxide via PEG-Catalase (HILP + PEG-Cat) reduced resistance artery FMD compared to high pressure exposure alone (HILP) in vessels obtained from exercised mice. *;P <0.01 at Δ 10, 20, 40, 60 and 100 cmH_2O. Data are presented as mean ± SE

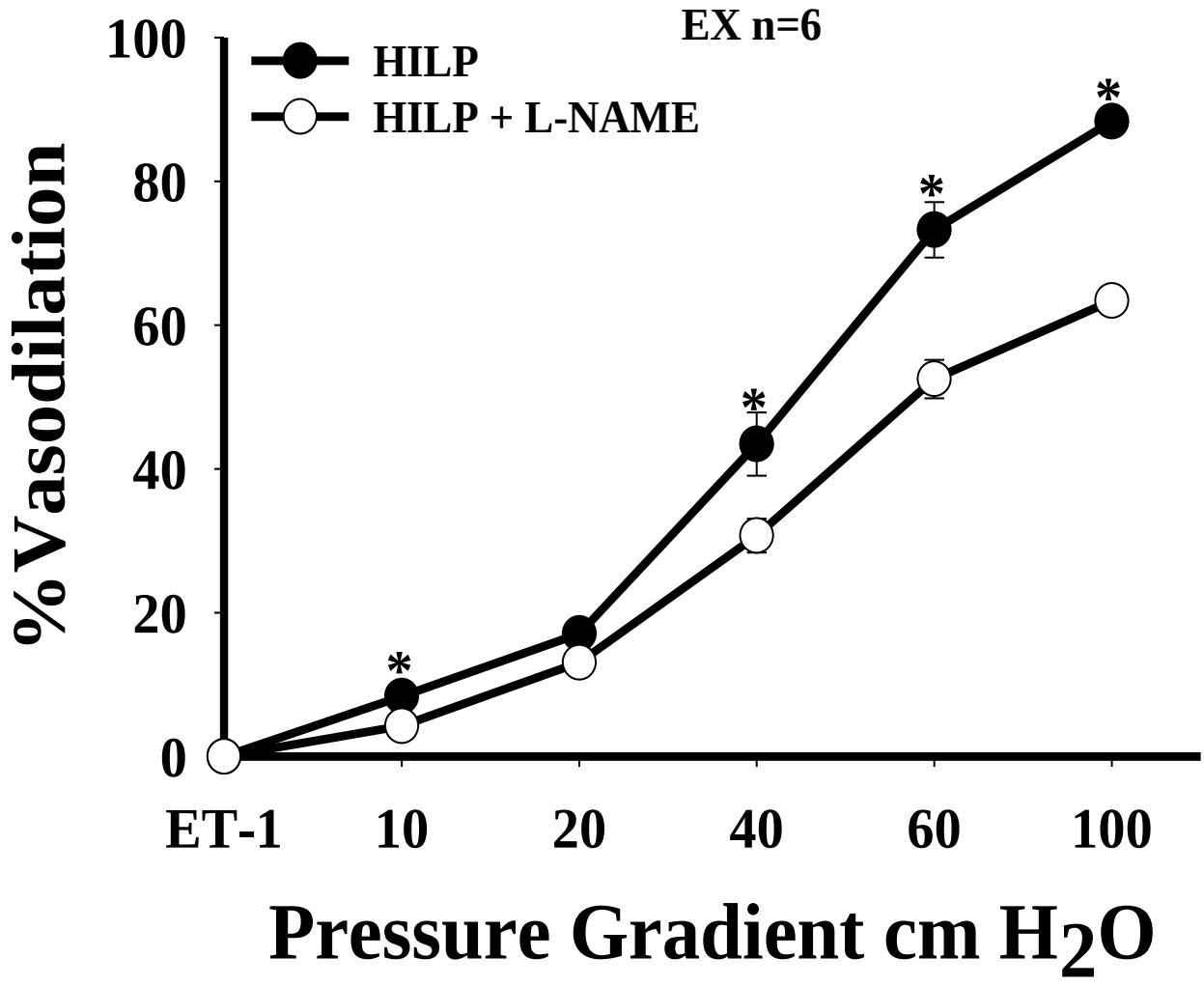

Figure 31 The effect of nitric oxide Synthase inhibition on post high intraluminal pressure resistance artery vasodilator function in arteries from exercised mice.

With high pressure, inhibition of $NO^{\cdot}$ production via L-NAME (HILP + L-NAME) reduced resistance artery FMD compared to high pressure alone (HILP) in vessels obtained from exercised mice. *;P <0.01 at Δ 10, 40, 60 and 100 cmH_2O. Data are presented as mean ± SE.

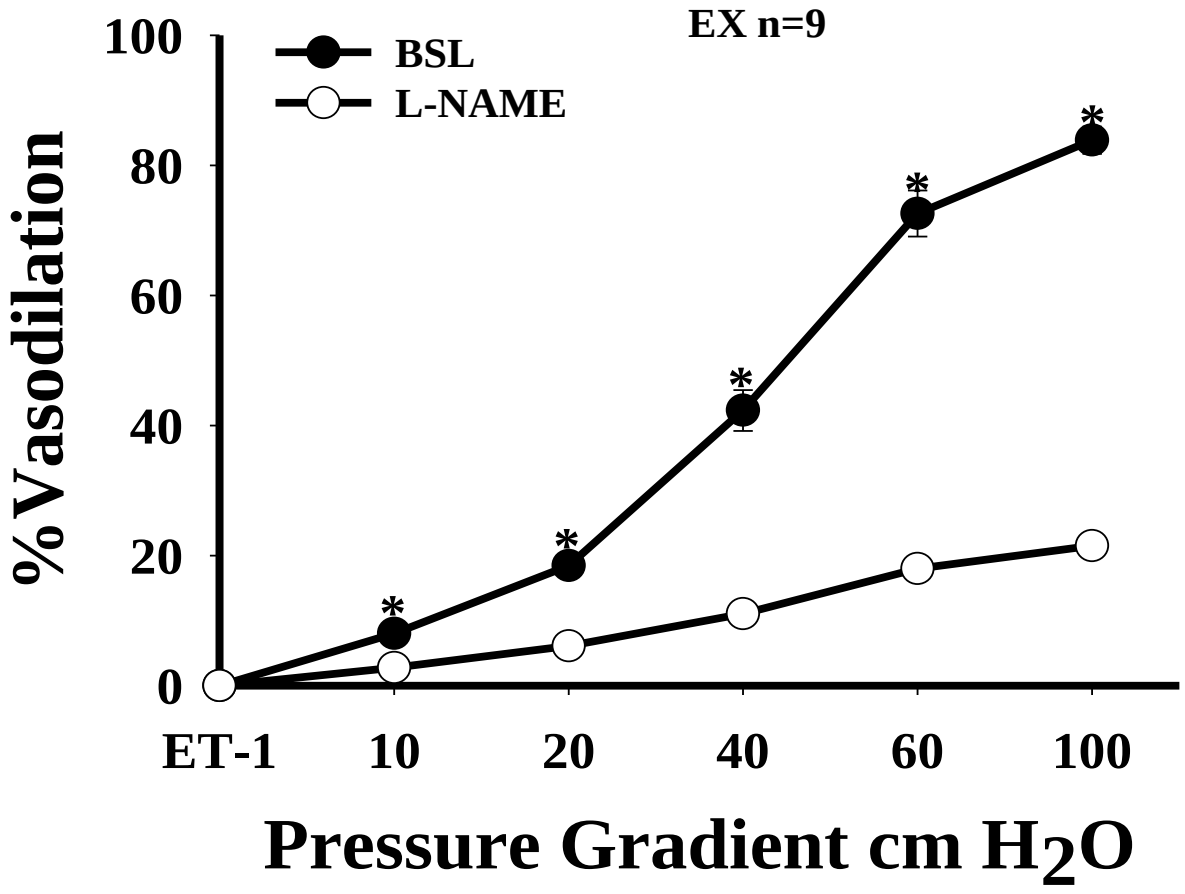

Figure 32 The effect of Nitric oxide Synthase inhibition on basal resistance artery vasodilator function in arteries from exercised mice.

At baseline (BSL) inhibition of $NO^{\cdot}$ production via L-NAME (L-NAME) reduced resistance artery FMD in vessels obtained from exercised mice. *;P <0.01 at Δ 10, 40, 60 and 100 cmH_2O. Data are presented as mean ± SE

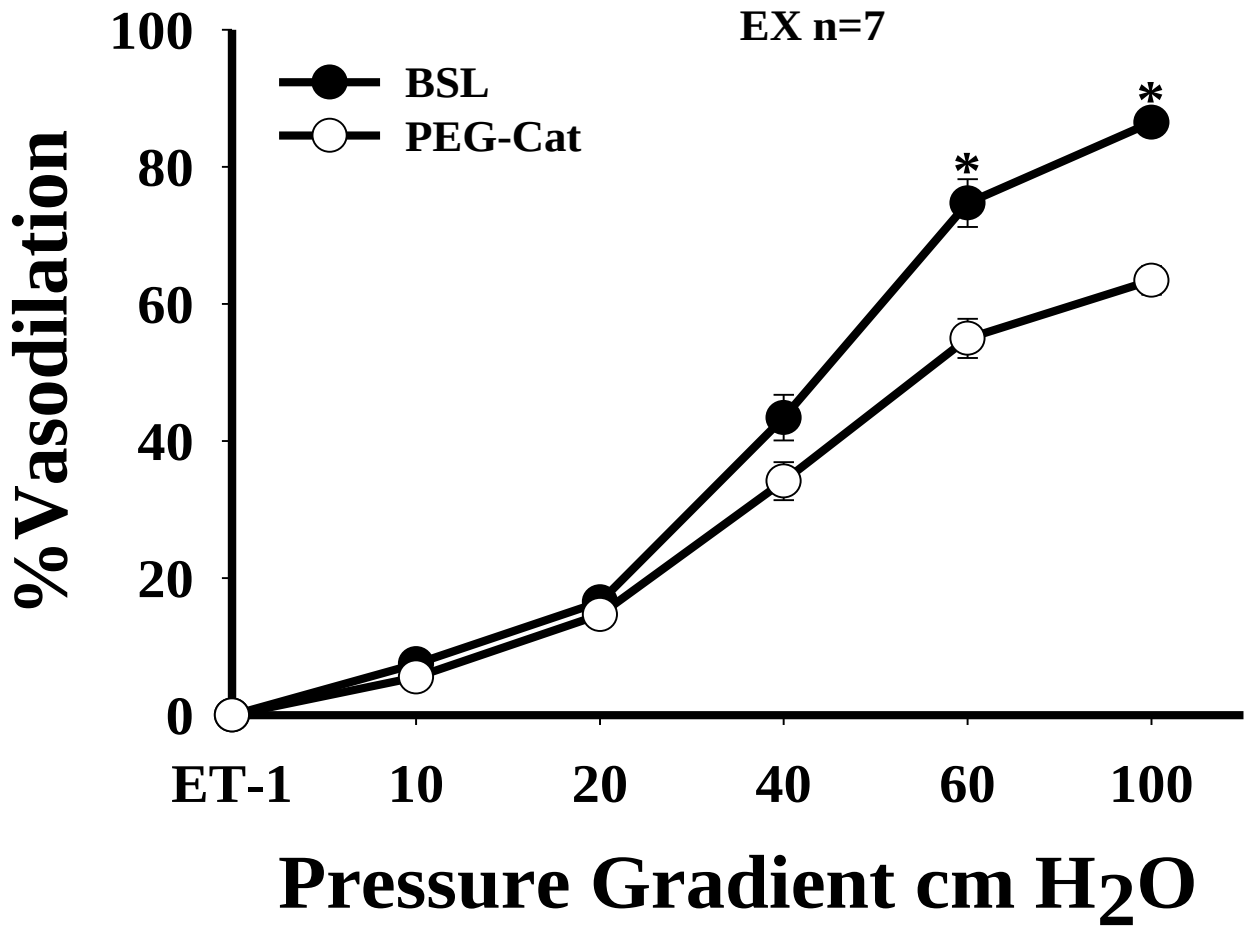

Figure 33 The effect of hydrogen peroxide scavenging on basal resistance artery vasodilator function in arteries from exercised mice.

At baseline (BSL), scavenging of hydrogen peroxide via PEG-Catalase reduced resistance artery FMD in vessels obtained from exercised mice. *;P <0.01 at Δ 60 and 100 cmH_2O. Data are presented as mean ± SE.

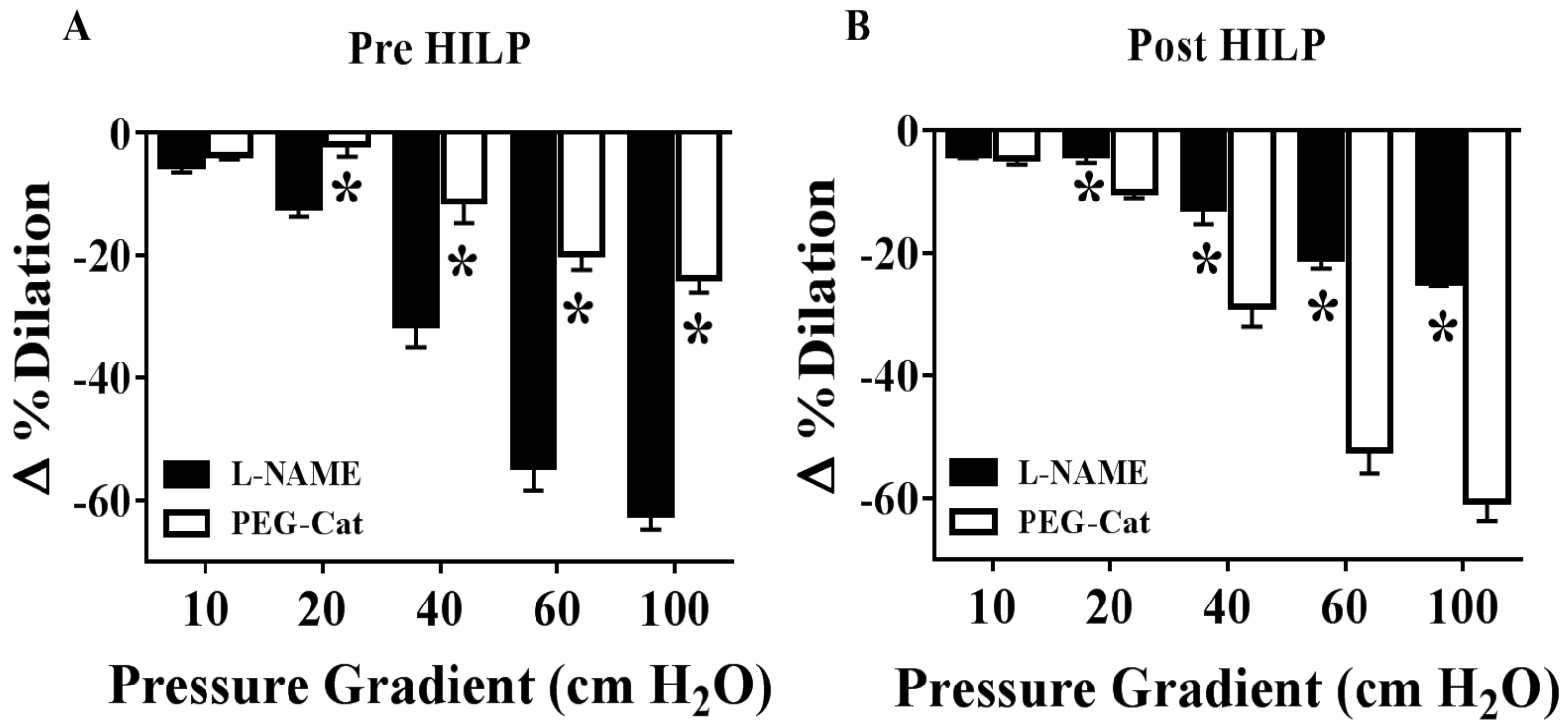

Figure 34 High intraluminal pressure elicits a phenotypic switch in resistance arteries obtained from exercised mice.

(A) In the basal condition L-NAME reduced microvessel FMD significantly more so than PEG-Cat. *;*P* <0.01 at Δ 20, 40, 60, and 100 cmH_2O. **(B)** Following exposure to high intraluminal pressure PEG-Cat reduced resistance artery FMD significantly more so than L-NAME. *;*P* <0.01 at Δ 20, 40, 60, and 100 cmH_2O. These findings indicate a switch from $NO^{\cdot}$ mediated dilation at rest to H_2O_2 mediated dilation following exposure to high intraluminal pressure. Data presented as Mean ± SE.

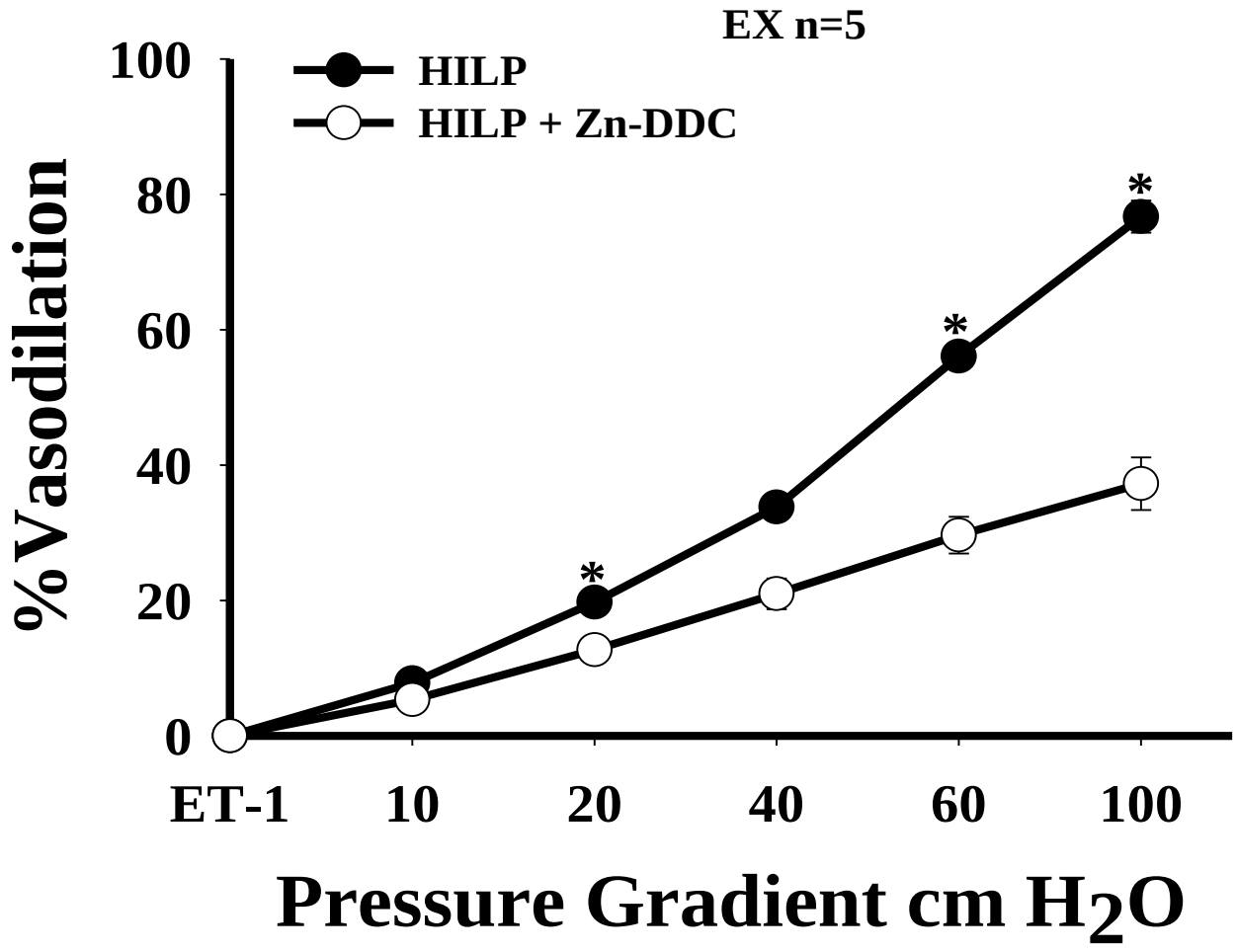

Figure 35 The effect of endogenous Superoxide Dismutase inhibition on post high intraluminal pressure resistance artery vasodilator function in arteries from exercised mice.

With high pressure, blockade of endogenous SOD with Zn-DDC (HILP + Zn-DDC) reduced resistance artery FMD compared to high pressure alone (HILP) in vessels obtained from exercised mice. *;P <0.01 at Δ 20, 60 and 100 cmH_2O. Data are presented as mean ± SE

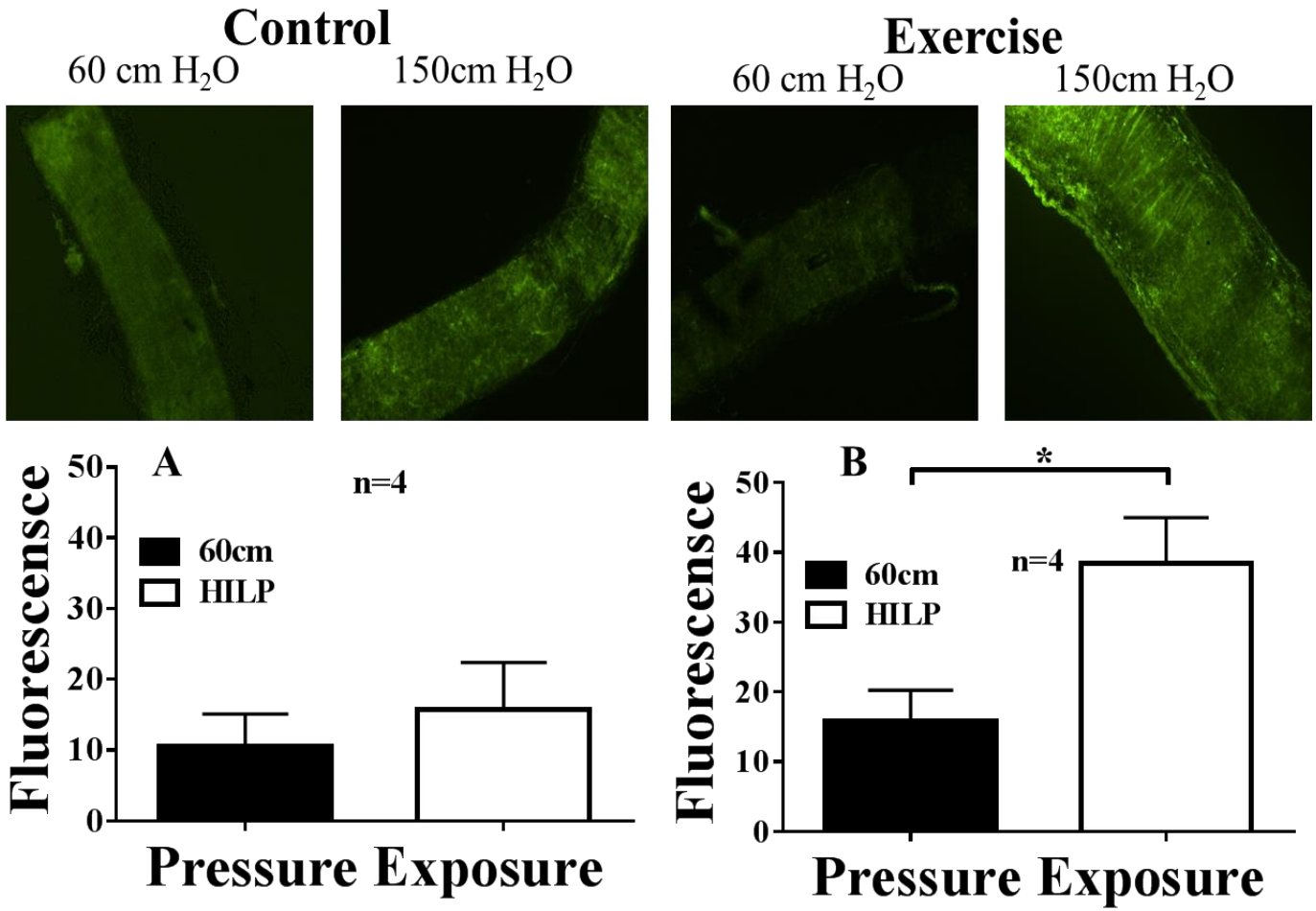

Figure 36 Influence of high intraluminal pressure on hydrogen peroxide production.

(A) In arteries obtained from control mice high pressure exposure (HILP) did not evoke a significant increase in hydrogen peroxide production compared to baseline (60 cmH_2O). **(B)** In arteries obtained from exercised mice high pressure exposure did elicit a significant increase in hydrogen peroxide production. *;*P* <0.05. Data presented as mean ± SE.

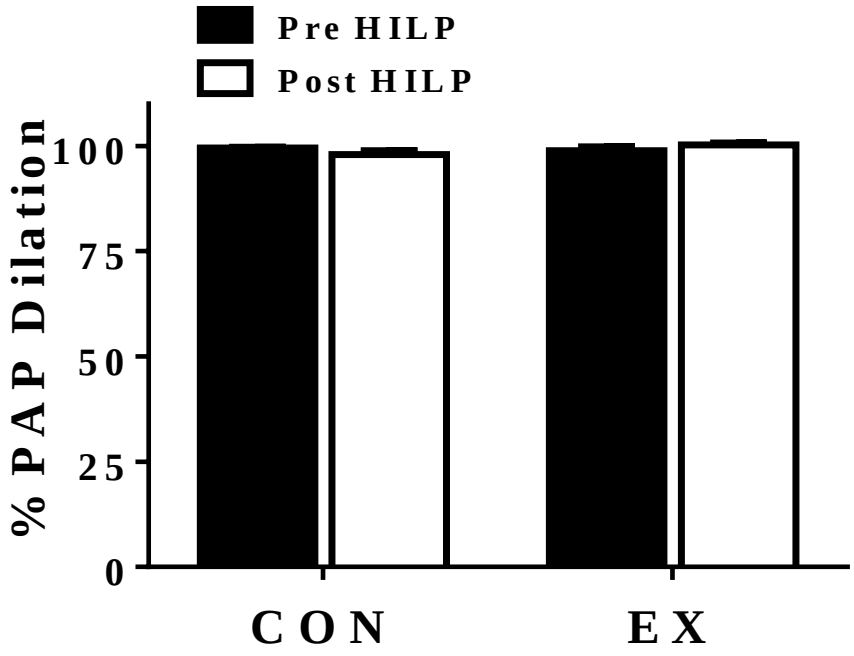

Figure 37 Papaverine mediated dilation in resistance arteries obtained from control and exercised mice.

No significant differences in Papaverine mediated dilation were observed between groups (CON vs EX) or within groups after exposure to high intraluminal pressure (Pre HILP vs Post HILP). Data presented as mean ± SE.

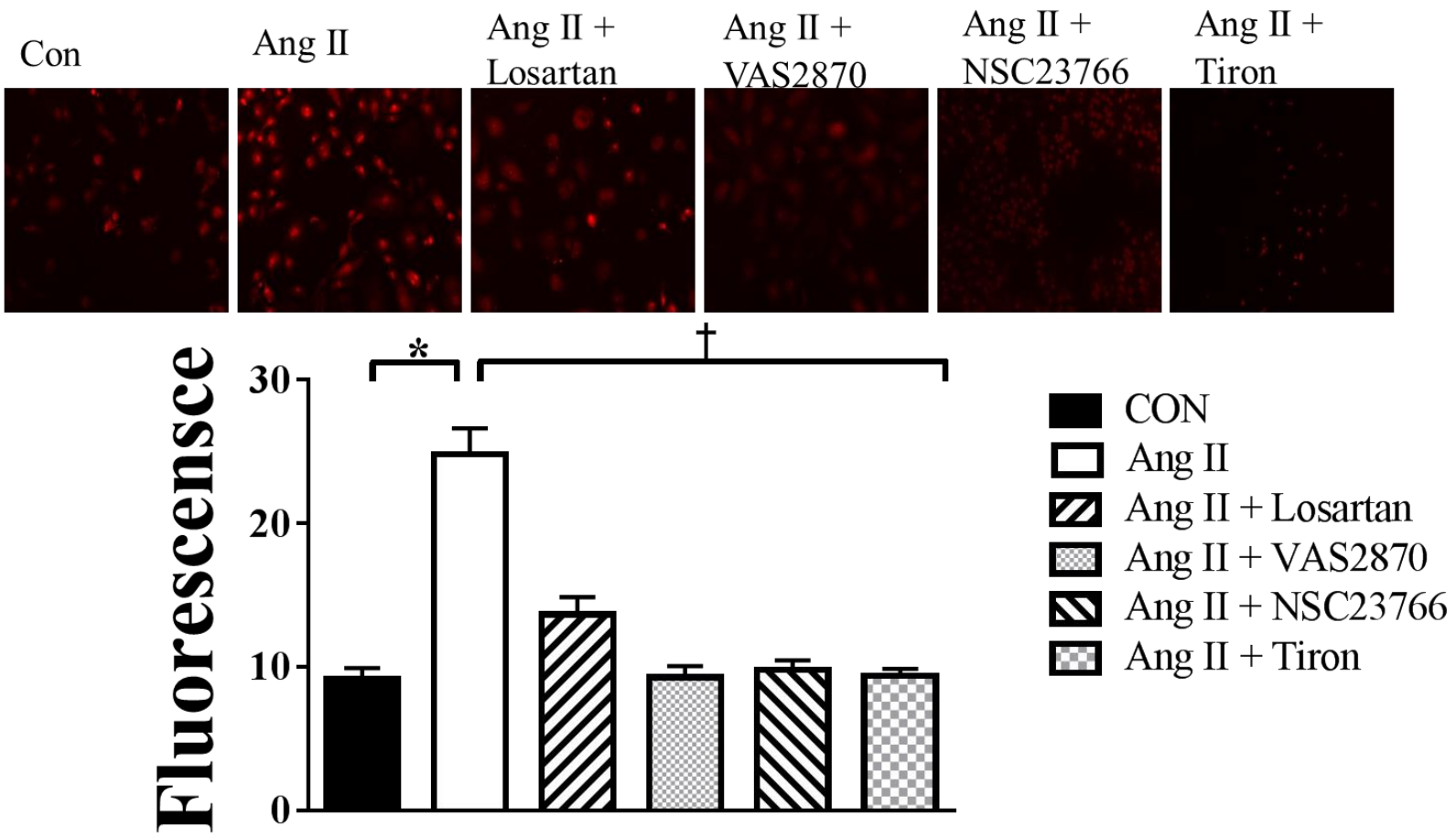

Figure 38 The influence Angiotensin II on superoxide production.

In HAMECs treatment with Ang II (400Nm) evoked a significant increase in superoxide production. *;*P* <0.05. Combined treatment with Ang II + Losartan (AT_1r blocker), VAS2870 or NSC23766 (NOX II inhibitors), or Tiron (SOD mimetic) significantly reduced the Ang II induced increase in superoxide production in HAMECS. †;P <0.05. Data presented as mean ± SE.

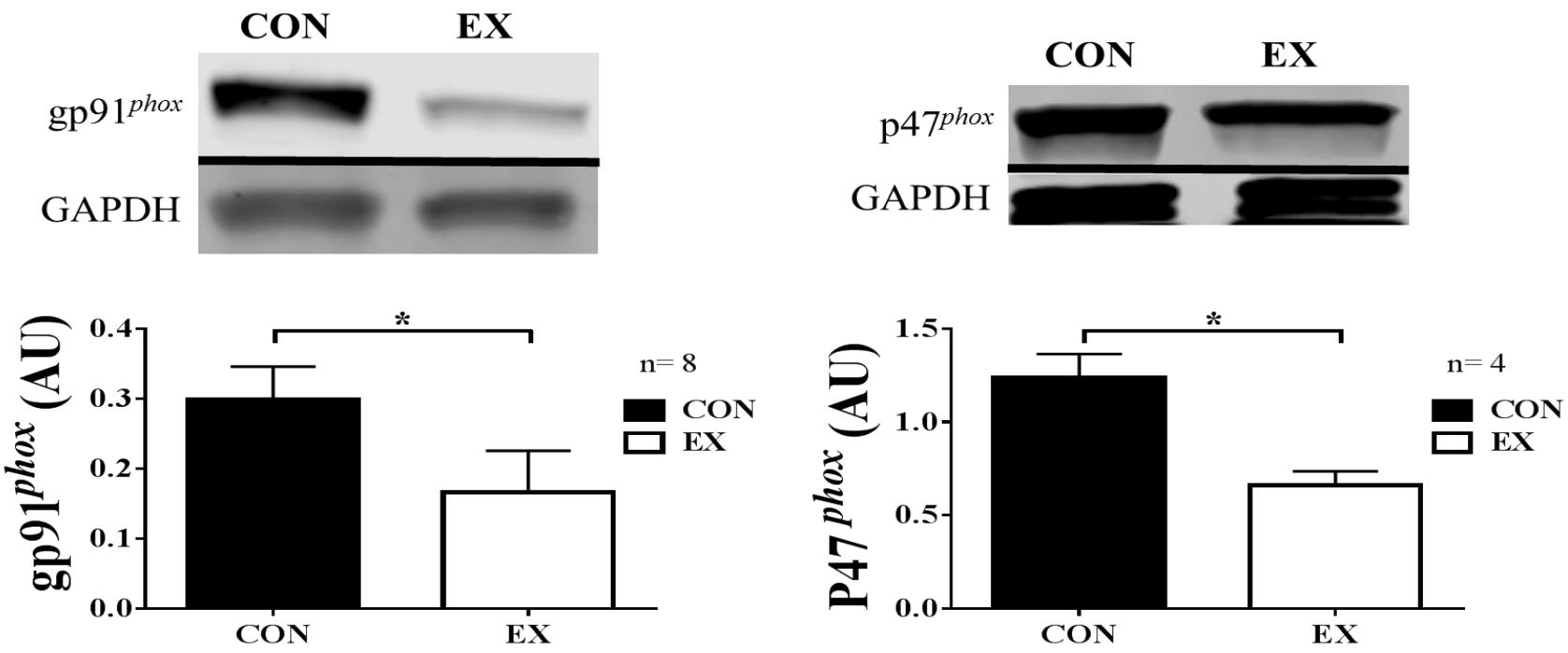

Figure 39 The influence of exercise on NADPH Oxidase II subunit expression.

(A) There was a significant reduction in gp91^{phox} expression in resistance arteries from exercised mice compared to resistance arteries from control mice. *;P <0.05. **(B)** There was a significant reduction in p47^{phox} expression in resistance arteries from exercised mice compared to resistance arteries from control mice. *;P <0.01. Data presented as mean ± SE.

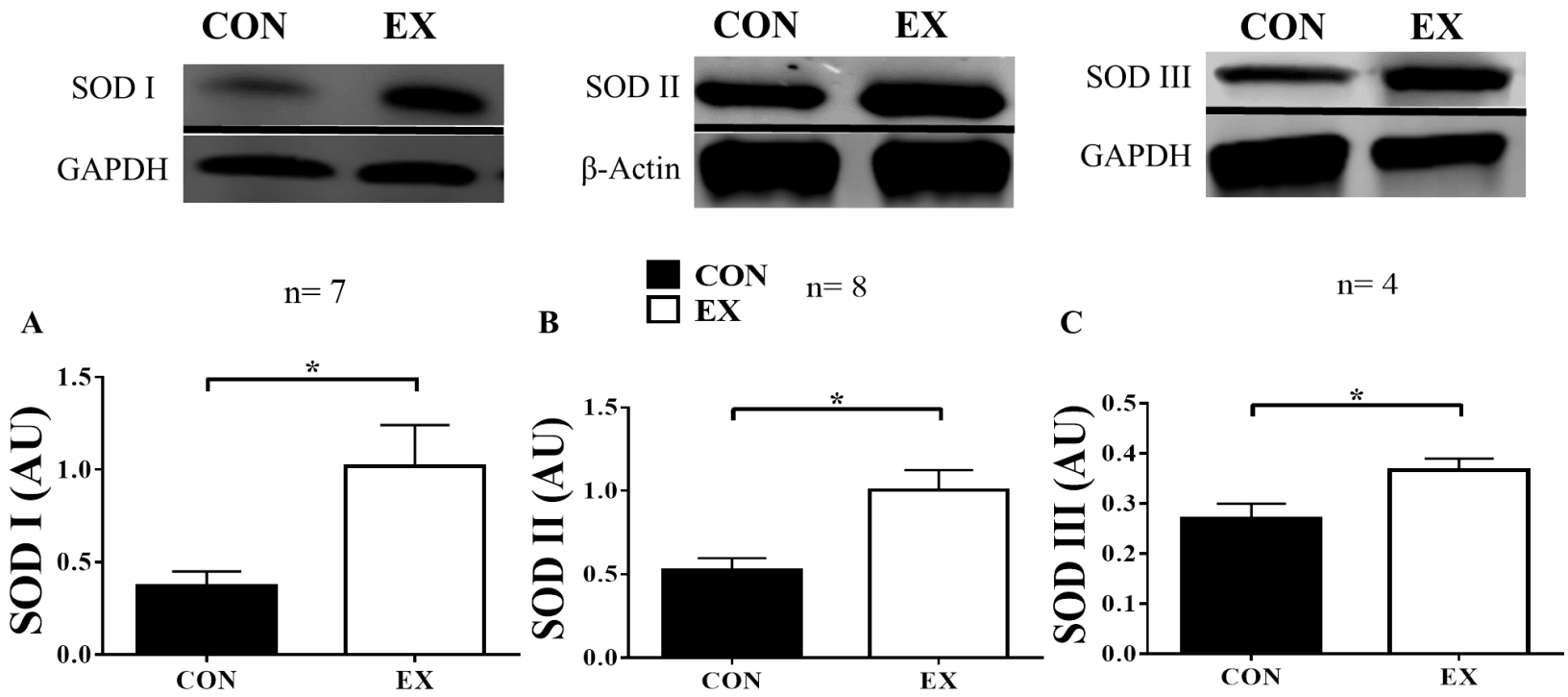

Figure 40 The influence of exercise on Superoxide Dismutase isoform expression.

(A) There was a significant increase in SOD I expression in resistance arteries from exercised mice compared to resistance arteries from control mice. *;P <0.01. **(B)** There was a significant increase in SOD II expression in resistance arteries from exercised mice compared to resistance arteries from control mice. *;P <0.01. **(C)** There was a significant increase in SOD III expression in resistance arteries from exercised mice compared to resistance arteries from control mice. *;P <0.05. Data presented as mean ± SE.

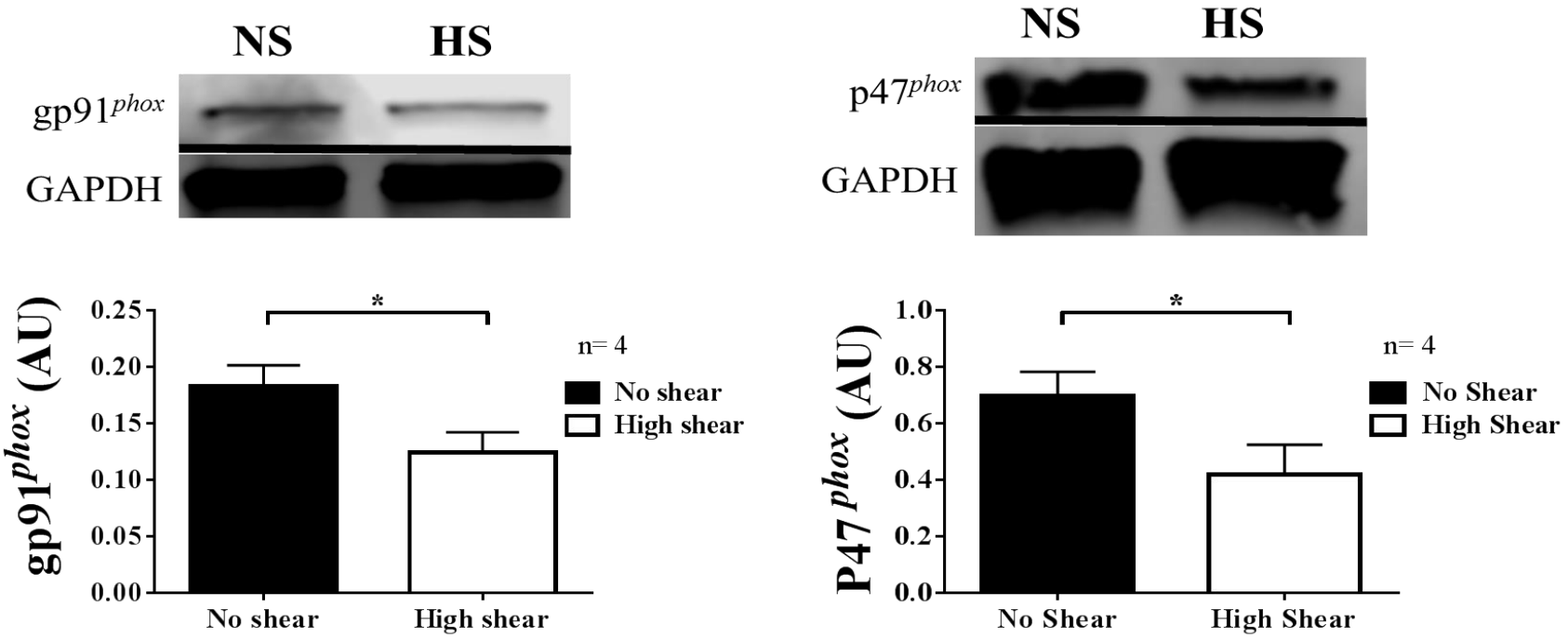

Figure 41 The influence of high shear stress on NADPH Oxidase II subunit expression in HAMECs.

(A) Exposure to high shear stress in HAMECs (HS) reduced gp91^{phox} expression compared to HAMECs raised under static conditions (NS). *;P <0.05. **(B)** Exposure to high shear stress in HAMECs reduced p47^{phox} expression compared to HAMECs raised under static conditions. *;P <0.01. Data presented as mean ± SE.

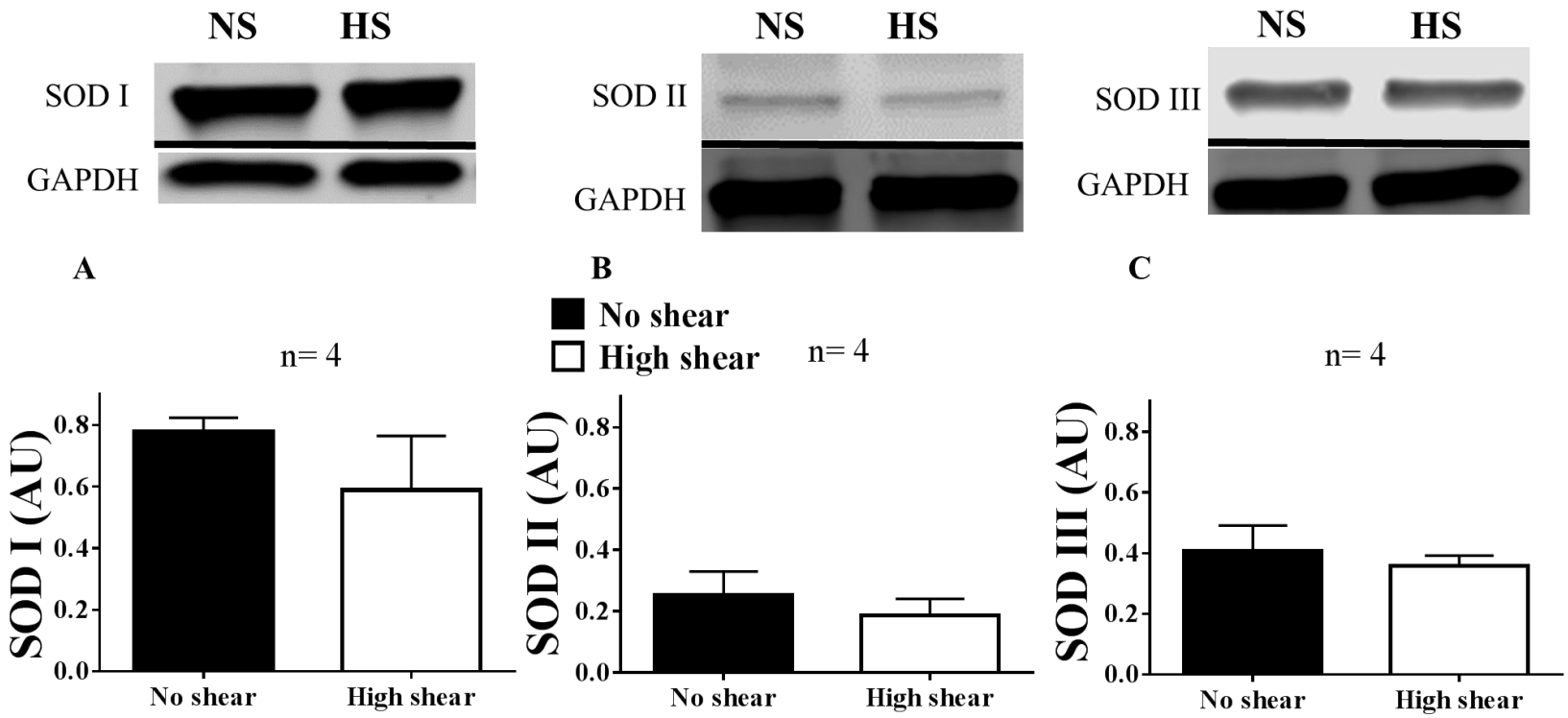

Figure 42 The influence of high shear stress on Superoxide Dismutase isoform expression in HAMECs.

(A) There was no effect of high shear stress in HAMECs (HS) compared to cells grown under static conditions (NS) in regards to SOD I expression or **(B)** SOD II expression or **(C)** SOD III expression. Data presented as mean ± SE.

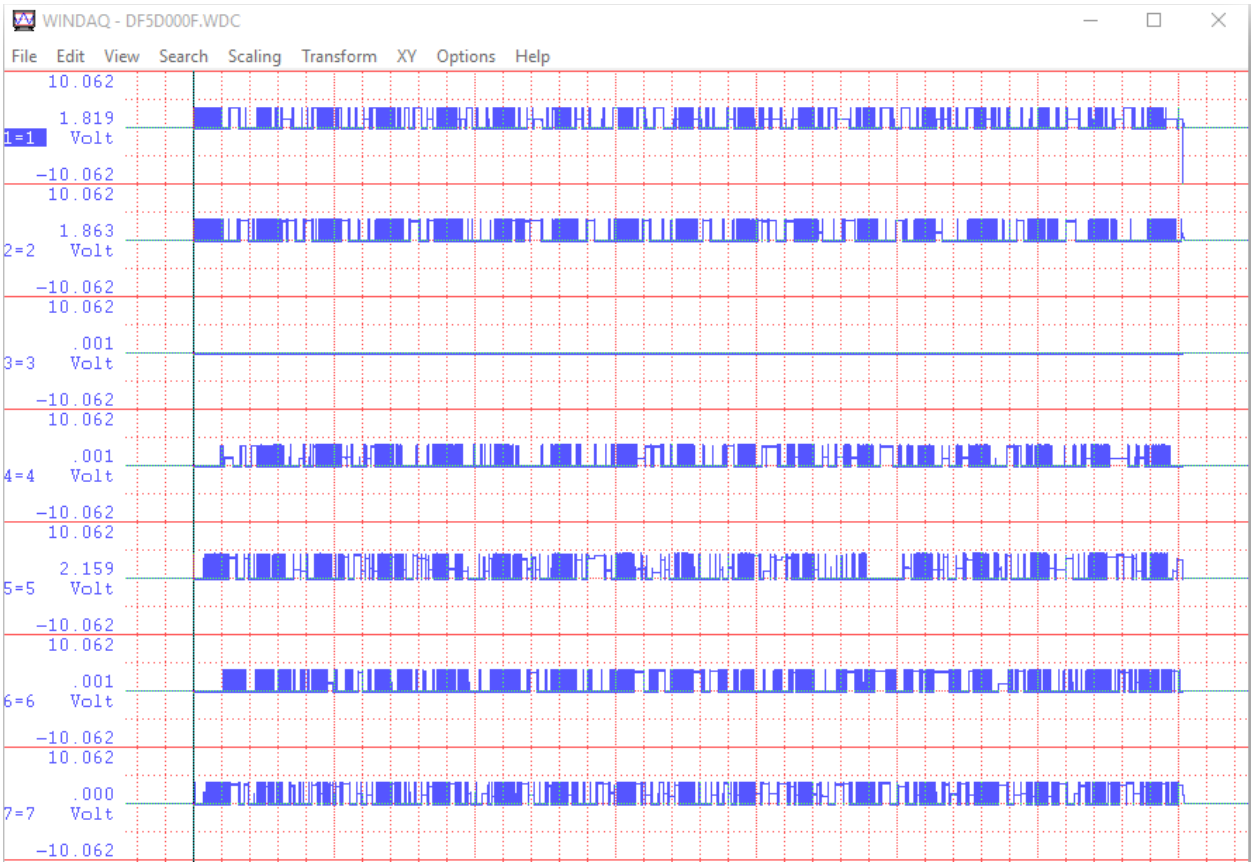

Figure 43 Activity patterns for voluntary wheel running mice.

Each block represents activity periods over one 24 hour time block. Down periods represent when mice were resting during the day. In the nocturne if we were to zoom in on the blocks we would see several peaks corresponding to individual revolutions of the running wheel. Each revolution is 40 cm and thus by counting the number of peaks over a 24 hour period we were able to compute the distance that mice ran every 24 hour period.

IV. D Discussion

The main findings from this study are that resistance arteries from exercised mice were protected from vascular dysfunction following high intraluminal pressure and this is likely due to an increase in SOD isoforms and a decrease in NOX II subunit protein expression. High intraluminal pressure induced resistance artery vasodilator dysfunction in a RAS/NOX II dependent fashion in control mice and resistance arteries from exercised mice were protected from this occurrence via H_2O_2 mediated dilation, as indicated by reduced dilation in response to catalase and inhibition of endogenous SOD. The reduction in preserved dilation with Catalase supports previous findings [35, 36], however the blockade of preserved dilation with endogenous SOD inhibition is novel.

High intraluminal pressure resulted in increased O_2^- production in resistance arteries from control mice, indicating a role for excess oxidative stress in high pressure induced vascular dysfunction. In contrast resistance arteries from exercised mice exposed to high intraluminal pressure did not produce an excessive O_2^- but produced an increased amount of H_2O_2. Resistance arteries from exercised mice were also found to express lesser amounts of NOX II subunits $gp91^{phox}$ and $p47^{phox}$ and greater amounts SOD I, SOD II, and SOD III protein relative to resistance arteries from control mice supporting previous findings of exercise adaptations in the vasculature. [173, 174]

Resistance arteries from control mice displayed greater vasoconstrictive sensitivity to Ang II and HAMECs treated with Ang II produced excessive O_2^- in a NOX II dependent fashion. Lastly HAMECs exposed to shear stress expressed lesser amounts of NOX II while SOD expression was unaffected. Taken together these findings suggest that resistance arteries from exercised mice were able to maintain flow mediated dilation following high pressure and the mechanism is related to

greater dismutation of O_2^- to H_2O_2, and a shear independent component of exercise may regulate endothelial SOD expression (i.e humoral factors).

Resistance arteries from exercise trained mice expressed less NOX II proteins and also produces less O_2^- when exposed to high intraluminal pressure compared to control mice. These findings may explain why L-NAME was still able to reduce vasodilation following high intraluminal pressure because increased O_2^- production leads to a subsequent reduction in $NO^{\cdot}$ bioavailability via $NO^{\cdot}$ quenching for peroxynitrate formation. [318, 319] Our finding of a more modest inhibition by exposure to Zn-DDC is likely explained by the fact that previous studies have found that Zn-DDC elicited dose-dependent inhibition of SOD with 75% inhibition at 10mM DDC. [320] Thus our blockade with 1mM likely resulted in only partial blockade of SOD I or II because of the dosage and likely would not have affected SOD II because of the cofactor specificity.

The mechanistic findings of the present study are in agreeance with previous reports of impaired vasodilator function in *ex vivo* arteries exposed to increased intraluminal pressure being rescued via angiotensin converting inhibitors or angiotensin receptor blockade [41]. In addition the protein kinase C (PKC) inhibitor, chelerythrine has also been shown to restore vasodilator function in response to high pressure indicating a PKC is a secondary messenger transducing the signal from the AT_1r to activation of NOX II [275]. Taken together these findings illustrate a role for a damaging pathway elicited by high pressure involving local activation of RAS, stimulation of PKC and potentially other messenger, and finally activation of NOX II which results excess oxidative stress in the microvascular wall. Although we did nt investigate the role of PKC here, our results suggest regular exercise may protect against angiotensin II induced vascular dysfunction by creating a favorable vascular redox environment which acts to blunt this pathway

via reduced NOX II expression and increased SOD expression in the vascular wall as found the study [173, 321, 322].

The importance of the findings in the present study lie in that several studies have linked high pressure-induced endothelium dysfunction to local vascular renin-angiotensin II production which leads to increased O_2^- production and a subsequent reduction in $NO^\cdot$ bioavailability [41, 187, 188, 234, 275]. This decrease in $NO^\cdot$ bioavailability likely contributes to the development of atherosclerosis as $NO^\cdot$ not only mediates vasodilation but has pleiotropic anti-atherogenic effect within the vasculature [323]. It also known that H_2O_2 can serve as a mediator of dilation in times of reduced $NO^\cdot$ bioavailability [39, 40, 292]. The present study indicates that an increase in vascular SOD may escalate the potential of this pathway during exercise training to preserve endothelium dependent vasodilation in response to elevated pressures during acute exercise.

Based on previous findings indicating exercise-like adaptations in the vasculature could be attributed to shear stress, we presumed shear stress in HAMECs would result in changes to both NOX II and SOD isoforms [21, 22]. Interestingly shear stress evoked a decrease in NOX II, similar to exercise but did not impact SOD levels. We now speculate that SOD expression may then be regulated by humoral factors associated with exercise rather than mechanotransduction factors. For example acute bouts of exercise are associated with small increases in Ang II and sub pressor doses (below those used in the present) are associated with increased expression of SOD. [324, 325]

Limitations to this study include that we were not able to distinguish if the molecular changes made in the vascular wall were specific to the endothelium as isolating the endothelium and quantifying protein expression solely form this vascular tunic was not feasible. As a substitution we used HAMECs and laminar shear stress to mimic exercise. Another limitation is

that we did not use a low shear control in addition to our static control which would have more appropriately mimicked *in vivo* conditions. We also did not perform high intraluminal pressure plus losartan experiments in resistance arteries from exercised mice in order to determine if inhibition of the AT_1R/PKC/NOX II prevented a phenotypic switch. We did not denude vessels in this particular study to determine if our findings were solely attributable to the endothelium although our group has done this in the past. [36] Lastly, in order to reduce variability within our data we used only male mice within a narrow age range. Future studies will need to be carried out to confirm our findings in the elderly and females.

In conclusion the results of the present study suggest that regular exercise may protect against high pressure induced vascular dysfunction in the adipose microcirculation by creating a favorable vascular redox environment and shear stress contributes to this in part. These results may be of importance for explaining how exercise can prevent endothelium dysfunction in response to acute challenges [38, 287, 326] and may be of importance for managing chronic hypertension induced endothelium dysfunction [275]. The ability of exercise to suppress oxidative stress and inflammation may help with obesity-driven aberrations in the adipose tissue microcirculation.

Chapter V: Summary of Results and Future Directions

The key findings of the human exercise intervention for this dissertation are that in previously sedentary, overweight and obese individuals regular aerobic exercise training prevented post-acute resistance exercise induced brachial artery and microvessel dysfunction. In addition the preserved microvessel vasodilator function in the exercise trained participants following acute exertion was H_2O_2 dependent.

The main findings from animal and cell study are that resistance arteries from exercised mice were protected from vascular dysfunction following high intraluminal pressure and this is likely due to an increase in SOD isoform and a decrease in NOX II subunit protein expression. The increase in SOD would explain why exercise vessels produce more H_2O_2 than vessels from sedentary counterparts. We also found that HAMECs exposed to laminar shear stress displayed a decrease in NOX II subunit protein expression. Taken together these findings suggest that shear stress may protect the adipose microcirculation from endothelium dysfunction mediated by high pressure, local RAS, and oxidative stress.

Our findings of protection against acute resistance exercise in both the brachial artery and adipose resistance vessels indicate that these findings may be systemic. However future studies using the leg press model as an acute stressor should include lower body conduit arteries, such as the popliteal artery. If our findings of an increase in SOD isoforms and a decrease in NOX II subunit protein expression in adipose resistance arteries from mouse apply to the systemic circulation this likely explains, at least in part, why regular exercisers are protected from a number of acute stressors, such as high fat feedings and mental stress. The difficulty of obtaining enough arteries from humans for molecular biology likely preclude these findings from being

replicated in human vessels. However pharmacological agents can be used in the future to elucidate the mechanisms through which H_2O_2 preserves dilation in human resistance arteries. The time course at which preserved vasodilation converts back to $NO^{\cdot}$ mediated dilation (from H_2O_2) also remains to be determined. With the field of inquiry on the acute of effects of exercise still relatively new, many exciting findings have yet to be discovered.

Appendix A

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Funds: $77,481

Source: The Microcirculatory Society
Title: Zweifach Student Travel Award (for Experimental Biology 2014)
Dates: 02/2014
Funds: $750

Source: International Society of Sports Nutrition
Title: The Effects of Inorganic Nitrate Supplementation on Macro and Microvasculature Function Following Acute Exercise
PI: Austin T. Robinson, MS; Shane A. Phillips PT, PhD (Faculty Sponsor)
Dates: 01/2014
Funds: $10,000

Source: The Microcirculatory Society
Title: Zweifach Student Travel Award (for Experimental Biology 2013)
Dates: 01/2013
Funds: $750

Not Funded:
Source: University of Illinois Graduate College; Chancellors Graduate Research Award
Title: Regular Aerobic Exercise Prevents Acute Hypertension -induced Vascular Dysfunction via Enhanced Oxidative Stress Buffering
PI: Austin T. Robinson, MS; Shane A. Phillips PT, PhD (Faculty Sponsor)
Dates: 09/2015
Funds: $4,000

RECOGNITIONS & HONORS:

05/2012	Graduated *Magna cum Laude* honors distinction with M.S. in Kinesiology from University of Illinois at Chicago - Chicago, Illinois (3.71/4.00)
05/2010	Graduated *Summa cum Laude* honors distinction with B.S. in Exercise Science from University of Indianapolis - Indianapolis, IN (3.92/4.00)
05/2010	Excellent Student in Exercise Science Award from University of Indianapolis, IN

PUBLICATIONS:

1. Hyperinsulinemia augments endothelin-1 protein expression and impairs vasodilation of human skeletal muscle arterioles. Mahmoud AM, Szczurek MR, Blackburn BK, Mey JT, Chen Z, **Robinson AT**, Bian JT, Unterman TG, Minshall RD, Brown MD, Kirwan JP, Phillips SA, and Haus JM. (Accepted: Physiological Reports 7-21-16)

2. Aerobic exercise training prevents acute exertion-induced vasodilator dysfunction in overweight and obese humans via hydrogen peroxide-mediated dilation. **Robinson AT**, Franklin NC, Norkeviciute E, Bian JT, Babana JC, Szczurek MR, and Phillips SA J Hypertens 2016 July;34(7):1309-16.

3. Circuit resistance training attenuates acute exertion-induced reductions in arterial function but not inflammation in obese women. Franklin NC, **Robinson AT**, Bian JT, Ali MM, Norkeviciute E, McGinty P, and Phillips SA. Metab Syndr Relat Disord. 2015 June;13(5):227-34.

4. Reduced flow-and acetylcholine-induced dilations in visceral compared to subcutaneous adipose arterioles in human morbid obesity. Grizelj I, Cavka A, Bian JT, Szczurek M, **Robinson AT**, Shinde S, Nguyen V, Braunschweig C, Drenjancevic I, and Phillips SA. Microcirculation. 2015 Jan;22(1):44-53.

5. Massage therapy restores peripheral vascular function following exertion. Franklin N, Ali M, **Robinson AT**, Norkeviciute E, and Phillips SA. Arch Phys Med Rehabil. 2014 Jun;95(6):1127-34.

PENDING PUBLICATIONS:

1. Aerobic exercise suppresses acute high intraluminal pressure induced oxidative stress in the adipose microvasculature. **Robinson AT**, Sudhahar V, Fancher I, Bian JT, Ali MM, Ushio-Fukai M, Levitan I, Fukai T, and Phillips SA. (In preparation)

2. Lower-extremity massage therapy attenuates reductions in local arterial function after eccentric resistance exercise. Franklin NC, Ali MM, **Robinson AT**, Norkeviciute E, and Phillips SA. (Submitted: Journal of Alternative and Complementary Medicine)

3. Mitochondrial depolarization contributes to restoration of flow-induced dilation of visceral adipose tissue resistance arteries in human morbid obesity. **Robinson AT**, Bian JT, Tsai PA, Szczurek M, Ali MM, Grizelj I, Cavka A, and Phillips SA. (In preparation)

4. Exercise protects against acute impairments in vascular function following a high sugar or high fat load. Das EK, Lai PY, Pleuss J, **Robinson AT**, Ali M, Gutterman DD, and Phillips SA. (In preparation)

ABSTRACTS:

1. Aerobic exercise dampens acute hypertension-induced vascular oxidative stress. **Robinson AT**, Fancher IS, Bian JT, Ali MM, Sudhahar V, Ushio-Fuaki M, Fuaki T, Levitan I, Phillips SA. Experimental Biology Conference April 2-6, 2016. San Diego, CA * Additional data added since November 2015 version.

2. NADPH Oxidase contributes to oxidative stress and reduced eNOS phosphorylation during hyperinsulinemia in human skeletal muscle arterioles and microvascular endothelial cells. Mahmoud AM, Ali MM, Miranda E, Mey JT, Blackburn BK, **Robinson AT**, Haus JM, Phillips SA. Experimental Biology Conference April 2-6, 2016. San Diego, CA

3. Aerobic exercise dampens acute hypertension-induced vascular oxidative stress. **Robinson AT**, Fancher IS, Bian JT, Ali MM, Sudhahar V, Ushio-Fuaki M, Fuaki T, Levitan I, Phillips SA. University of Illinois at Chicago College of Applied Health Sciences Research Day November 4, 2015. Chicago, IL

4. Rotenone-induced mitochondrial depolarization in endothelial cells results in in vasodilation via H_2O_2 dependent NO production. Urquhart M, **Robinson AT**, Tsai PA, Bian JT, Szczurek M, Phillips SA. Chicago Area Undergraduate Research Symposium April 11, 2015. Chicago, IL

5. Weight loss with low carbohydrate diets improves flow induced vasodilation in resistance arteries. Bian JT, Szczurek M, Ranieri C, Grizelj I, Cavka A, **Robinson AT**, Marsh G, Li K, Sanyaoulu RA, Shinde S, Phillips SA. American Heart Association Scientific Sessions November 15-19, 2014. Chicago, IL

6. Hyperinsulinemia disrupts vascular homeostasis, enhances oxidative stress and impairs flow induced dilation of human skeletal muscle arterioles. Mahmoud AM, Szczurek M, **Robinson AT,** Mey JT, Blackburn BK, Bian JT, Brown MD, Phillips SA, Haus JM. American Heart Association Scientific Sessions November 15-19, 2014. Chicago, IL

7. The restorative effect of apocynin on flow induced dilation of arterioles involves H_2O_2 and the activation of Rho A/Akt pathways and IK/SK channels in human obesity. Bian JT, Szczurek M, **Robinson AT**, Marsh G, Li K, Tsai PA, Phillips SA. 11th International Symposium on Resistance Arteries (ISRA) September 7-11, 2014, Banff, AB, Canada

8. Hyperinsulinemia augments endothelin-1 release and impairs vasodilation of human skeletal muscle arterioles. Mahmoud AM, Mey JT, Blackburn BK, Coleman K, Somal VS,

Bian JT, Szczurek M, **Robinson AT**, Phillips SA, Haus JM. American Diabetes Association Scientific Sessions June 13-17, 2014. San Francisco, CA

9. Aerobic exercise training reverses reduced endothelial function after acute exertion in overweight adults. **Robinson AT**, Norkeviciute E, Phillips SA. ACSM Annual Meeting May 27-31, 2014 Orlando, FL

10. Aerobic exercise training protects against impaired microvasculature function following acute exertion by enhancing hydrogen peroxide-mediated dilation in overweight adults. **Robinson AT**, Szczurek M, Bian JT, Phillips SA. Experimental Biology Conference April 26-30, 2014. San Diego, CA

11. Hypocaloric, but not isocaloric, low fat diets improve microvascular nitic oxide dependent vasodilation in obese subjects. Szczurek M, Bian JT, Ranieri C, Grizelj I, Cavka A, **Robinson AT**, Marsh G, Phillips SA. American Heart Association Scientific Sessions November 17-20, 2013. Dallas, TX

12. Massage therapy induced protection against systemic endothelial functional impairment following exertional muscle damage is associated with a reduction in systemic inflammation. **Robinson AT**, Franklin NC, Ali MA, Norkeviciute E, Baynard T, Phillips SA. American College of Sports Medicine Midwest Chapter 41st Annual Meeting November 8-9, 2013. Merrillville, IN

13. Massage therapy improves systemic vascular endothelial function after exertion-induced muscle injury. Franklin NC, Ali MM, Norkeviciute, E, **Robinson AT**, Phillips SA. ACSM, Annual Meeting. May 27-31, 2013. Indianapolis, IN

14. Impaired vasodilation of human obese visceral resistance arteries is improved by apocynin through production of hydrogen peroxide and activation of SK and IK channels. Bian JT, Szczurek M, **Robinson AT**, Phillips SA. Experimental Biology Conference April 20-24, 2013. Boston, MA.

15. Mitochondrial reactive oxygen species contribute to impaired flow-induced dilation in visceral but not subcutaneous adipose tissue resistance arteries in human obesity. **Robinson AT**, Szczurek M, Bian JT, Cavka A, Grizelj I, Phillips SA. Experimental Biology Conference April 20-24, 2013. Boston, MA.

INVITED TALKS:

1. "Concise review of Exercise Physiology" Benedictine University, Master of Science in Clinical Exercise Physiology Program. Host: Pedro Del Corral, MD, PhD. June 14, 2016

2. "Aerobic Exercise Modulates the Redox Environment in the Adipose Microvasculature" University of Wisconsin-Madison, Department of Kinesiology. Host: William Schrage, PhD. May 26, 2016

3. "Aerobic Exercise Modulates the Redox Environment in the Adipose Microvasculature" University of Texas at Arlington, Department of Kinesiology. Host: Paul Fadel, PhD. May 23, 2016

TEACHING:

Benedictine University- Lisle, IL

- Course instructor for EXPH 662 Advanced Exercise Physiology II- Lecture and facilitate discussion amongst graduate students in Advanced clinical topics in the field of Exercise Physiology 08/2015 – 12/2015

University of Illinois Chicago- Chicago, IL

- Lab Instructor for KN 251/252 Human Anatomy and Physiology- Teach students anatomical structures using models, cadavers, and electronic media 8/2015 - 05/2016
- Lab Instructor for KN 452 Advanced Exercise Physiology – Guided students in field-related research, experimental design, literature review, hypothesis formation, and implementation 08/2011 - 05/2012
  Lab Instructor for KN 352 Principles of Exercise Physiology – Instructed students on use of basic equipment used in the research setting 06/2011 - 08/2011
- Lab Instructor for KN 343 Advanced Exercise Assessment - Instructed students on proper methods and modalities used in clinical setting and for basic fitness assessment 01/2011 – 05/2011
- Class Instructor for KN 137 Aerobic Conditioning - Instructed an activities-based group fitness class. 08/2010 – 05/2011

MENTORING:

Po-An Tsai, Master of Science in Rehabilitation Sciences, May 2015. M.S. project: Mitochondrial depolarization improves nitric oxide bioavailability in the visceral adipose endothelium

James Babana, Bachelors of Science in Kinesiology, May 2015. Capstone Project: Aerobic exercise training mitigates the impact of acute exertion on peripheral vascular dilatory function in overweight adults. Presented: Babana J, Robinson AT, Phillips SA. UIC Student Research Forum; April 2, 2015

Megan Urquhart, Bachelors of Science in Biology, May 2015. Capstone Project: Rotenone-induced mitochondrial depolarization in endothelial cells results in in vasodilation via H_2O_2 dependent NO production. Presented: Urquhart M, Robinson AT, Tsai PA, Bian JT, Szczurek M, Phillips SA. Chicago Area Undergraduate Research Symposium; April 11, 2015

CERTIFICATES & MEMBERSHIPS:

04/2015	Applied Health Sciences Student Council (UIC)
10/2014	Student Membership- American Heart Association (AHA)
12/2013	Student Membership- International Society of Sports Nutrition (ISSN)
11/2012	Student Membership - Microcirculatory Society (MCS)
10/2012	Student Membership - The American Physiological Society (APS)

08/2012	University of Illinois at Chicago Graduate Student Council (GSC); (08/2014) Appointed as GSC Finance Sub Committee member
10/2010	American Heart Association certification in CPR & AED
05/2010	National Strength & Conditioning Association (NSCA) Certified Strength & Conditioning Specialist (CSCS)
11/2008	National Strength & Conditioning Association (NSCA) Certified Personal
Trainer	(CPT)
08/2008	Red Cross certification in First Aid; Emergency Responder
01/2008	University of Indianapolis Exercise Science Club
07/2007	Student Member - National Strength & Conditioning Association