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EXERCISE MITIGATES THE EFFECTS OF HYPERHOMOCYSTEINEMIA
ON ADVERSE SKELETAL MUSCLE REMODELING

By

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B.A., Purdue University, 2008
M.S., University of Louisville, 2010
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A Dissertation

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The School of Medicine of the University of Louisville

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DEDICATION

This dissertation is dedicated to my mother, Ms. Linda Winchester, for supporting me and encouraging me throughout my entire life.

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I would like to thank my mentor, Dr. Suresh C. Tyagi for his continuous support and encouragement throughout my time in his laboratory. His mentorship and passion for scientific understanding are unsurpassed. I would also like to thank him for believing in me during unfortunate circumstances during my doctoral program; it is truly appreciated. I am grateful to Dr. Schuschke for serving as my co-mentor and for his continuous guidance throughout my research endeavors. I would like to thank Dr. Joshua for providing valuable insight into the details of my dissertation project and for providing valuable advice to facilitate my doctoral studies. I would like to thank Dr. Stremel for serving on my committee and for elevating my personal understanding of physiology to new heights. I would like to thank Dr. Bratcher for serving on my committee and for all of her support, advice and friendship over the years. I would like to extend my most sincere thanks to all of the professors of the Department of Physiology for their advice, support and remarkable academic instruction. I would like to thank the administrative staff at the Department of Physiology for all of their assistance over the years.

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ABSTRACT

EXERCISE MITIGATES THE EFFECTS OF HYPERHOMOCYSTEINEMIA ON ADVERSE SKELETAL MUSCLE REMODELING

Lee Winchester

August 3, 2015

Background: Hyperhomocysteinemia (HHcy) is heavily implicated in diabetes and cardiovascular disease. HHcy is known for causing inflammation and vascular remodeling, particularly through production of reactive oxygen species (ROS) and matrix metalloproteinase-9 (MMP-9). Although its impact on skeletal muscle is rather unclear, HHcy is known to cause skeletal muscle weakness and functional impairment. The impact of HHcy on inflammation makes it seem likely that HHcy causes skeletal muscle fibrosis through induction of inflammatory factors and destructive macrophages. Exercise has been shown to reduce homocysteine levels and therefore, could serve as a promising intervention for HHcy. The purpose of this study was to investigate whether HHcy causes

skeletal muscle fibrosis through induction of inflammation and determine if exercise can mitigate these effects.

Methods: For these experiments we used J774A.1 and Raw 264.7 macrophages in cell culture and C57, CBS+/-, FVB, MMP-9 -/-, and CBS/MMP-9 double KO mice for animal models. A 6 week treadmill exercise protocol was used as intervention for HHcy. Arterial blood pressure measures were taken through tail-cuff method. Hind limb perfusion was measure via laser Doppler. Plasma Hcy estimations were measured thorough dot blot. Measurement of skeletal muscle or macrophage protein expression occurred through the use of western blot, immunocytochemistry or immunohistochemistry. Levels of skeletal muscle mRNA expression were determined through PCR and qPCR. Collagen deposition in the skeletal muscle was measured using Masson's trichrome staining. ROS production in macrophages was measured using a DCFDA assay.

Results: In CBS+/- mice, increased plasma Hcy levels were associated with decreased body weight and muscle mass, femoral artery perfusion, femoral artery lumen diameter and oxidative metabolism. These mice displayed increased wall to lumen ratio, mean arterial blood pressure, collagen deposition and elevated myostatin protein expression. It was determined that CBS/MMP-9 mice did not display any of these conditions. Exercise was capable of mitigating all of these effects in CBS +/- mice.

Skeletal muscle from CBS+/- mice had elevated markers of inflammation and hypoxia including VEGF, iNOS, EMMPRIN, MMP-9, and IL-1 β . Cell culture

studies determined that Hcy caused macrophages to shift towards a destructive, M1 phenotype as indicated by elevated CD40, ROS, EMMPRIN and MMP-9 production. EMMPRIN inhibition prevented induction of CD40 and ROS. In CBS +/- mice, it was determined that HHcy causes increased elevations in F4/80 and TNF- α expression, which are indicative of M1 macrophages. Exercise was capable of reducing the M1 macrophage population.

Conclusions: We conclude that HHcy causes skeletal muscle fibrosis through induction of MMP-9 and M1 macrophages. Exercise is capable of mitigating the pathologies associated with HHcy.

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CHAPTER I

INTRODUCTION

Homocysteine (Hcy) is a non-protein forming, non-essential amino acid that is derived from methionine. Homocysteine is normally present in healthy, human plasma, but high levels of plasma Hcy, termed Hyperhomocysteinemia (HHcy), has been strongly associated with diseases such as diabetes mellitus, cardiovascular diseases and skeletal muscle dysfunction. It is well established that HHcy is directly correlated with aortic stiffness, decreased renal function, endothelial dysfunction and hypertension (Bortolotto et al., 1999; Givvimani, Pushpakumar, Veeranki, & Tyagi, 2014; Naess, Nyland, Idicula, & Waje-Andreassen, 2013; Sutton-Tyrrell, Bostom, Selhub, & Zeigler-Johnson, 1997; Wiernicki, Millo, Safranow, Gorecka--Szyld, & Gutowski, 2011). However, more recent evidence suggests that HHcy also causes damage to skeletal muscle. Studies have shown that HHcy is strongly correlated with decreased muscle strength and functional limitations as well as skeletal muscle damage (Miller et al., 2000; Swart et al., 2013; S. Veeranki, Winchester, & Tyagi, 2015). Children and animals born with a mutation that causes hyperhomocysteinemia due to the inability to properly metabolize homocysteine display lower body weights and decreased muscle mass compared to their healthy counterparts (Kalra, Ghose, & Sood, 1985; Kanwar, Manaligod, & Wong, 1976).

Many years of research on HHcy have sought to determine the mechanisms through which it causes these deleterious effects. The ability of HHcy to induce high levels of reactive oxygen species (ROS) indicates that Hcy may inflict a great deal of damage through increased oxidative stress and inflammation (Hayden & Tyagi, 2004; Signorello et al., 2007; S. C. Tyagi, Lominadze, & Roberts, 2005). HHcy is also known to increase the expression and activity of matrix metalloproteinases (MMP), particularly MMP-9 (Seung Jin Lee et al., 2012; Moshal et al., 2006; Neetu Tyagi et al., 2009). MMP-9 is an endoproteinase that degrades components of the extracellular matrix (ECM). This degradation of the ECM can lead to adverse remodeling in muscle tissues.

It is commonly known that macrophages play a pivotal role in the inflammatory response and the progression of diseases such as atherosclerosis. More recent evidence has suggested that HHcy may have an effect on macrophage migration and function as well. Research has demonstrated that HHcy increases macrophage expression of MMP-9, vascular endothelial growth factor (VEGF) and monocyte chemoattractant protein-1 or MCP-1 (Kiriakidis et al., 2003; Seung Jin Lee et al., 2012; Wang, Siow, & Karmin, 2001). The ability of Hcy to alter the expression of certain macrophage proteins may evolve as another central mechanism through which HHcy causes disease progression.

The concept that regular exercise provides healthful benefits and plays a rather ubiquitous role in the prevention and reversal of many diseases is rather concrete. However, the effect of regular exercise on HHcy is not very well established. Recent studies have demonstrated that exercise can lower plasma

homocysteine levels and mitigate the adverse effects of HHcy in many organs (Hrncic et al., 2013; König et al., 2003; Neuman, Albright, & Schalinske, 2013; Randeva et al., 2002; Vincent, Bourguignon, & Vincent, 2006). In the current study, we investigated the effects of HHcy on skeletal muscle remodeling and inflammation as well as the ability of exercise to attenuate these effects.

CHAPTER II

BACKGROUND

Skeletal Muscle

Function of Skeletal Muscle

Skeletal muscle is a high-energy consuming tissue that makes up roughly 40 to 50% of the total bodyweight in the average human (Powers & Howley, 2007). It consists of very long, multinucleated cells called myocytes that are bundled up and surrounded by connective tissues. In general, skeletal muscle has 2 primary functions: 1) production of contractile force that provides breathing, locomotion and postural support; and 2) thermogenesis during periods of cold stress. For force production, depending on the basic function of the muscle it may consist of either fast twitch (type I) fibers or slow twitch (type II) fibers (Powers & Howley, 2007). Type I fibers are used for repetitive, low-intensity functions, such as postural support. These fibers provide less force generation, but are fatigue resistant and have a higher reliance on oxidative metabolism and therefore have a greater density of mitochondria and oxidative enzymes. Type II fibers can be further divided out into two fiber subsets: type IIx and type IIa (Powers & Howley, 2007). Type IIx fibers are very glycolytic and produce great contractile force, but are susceptible to fatigue due to a lower mitochondrial

density. Type IIa fibers are an intermediate fiber type. They produce a moderate contractile force and are more fatigue resistant than Type IIx fibers.

Anatomy and Physiology of Skeletal Muscle

The outermost layer of a skeletal muscle is the epimysium, which is a connective tissue that surrounds the entire muscle. Inward from the epimysium is the perimysium, which surrounds individual bundles of myocytes, called fascicles. Inside of these fascicles are individual myocytes that are surrounded by a connective tissue called the endomysium (Powers & Howley, 2007). Just under the endomysium lies another protective layer called the basement membrane. Below the basement membrane is the myocyte cell membrane, called the sarcolemma. Within the sarcolemma lies the intracellular fluid called sarcoplasm.

Myocytes, unlike most other cell types, contain multiple nuclei and are striated because of contractile segments called sarcomeres. Within each myocyte are structures called myofibrils, which contain the contractile proteins of the muscle, called myosin and actin (Powers & Howley, 2007). On the actin filament itself lies two additional proteins called troponin and tropomyosin, which are used for contractile regulation. The striated regions, called sarcomeres, are within the myofibrils and consist of areas of myosin/actin overlap, as well as an area where myosin does not overlap with actin, called the H zone. Sarcomeres are divided by a wall of structural proteins, which make up a region of the sarcomere called the Z line. During contraction, with required energy coming

from the dephosphorylation of adenosine triphosphate (ATP), Myosin heads “cock” and form crossbridges with actin and then pivot inward towards the H zone. This process is called the “sliding filament theory” and is evident by the decrease in sarcomere length from Z line to Z line.

Surrounding each myofibril is a network of channels that serves as a storage site for calcium, which is crucial for regulation of muscular contraction (Powers & Howley, 2007). This structure is called the sarcoplasmic reticulum. The transverse tubules are another set of channels that pass through the muscle fiber. Each myocyte is connected to a branch of a motor neuron through a neuromuscular junction, which serves as the site of electrochemical transmission. Collectively, all of the myocytes that are innervated by a single motor neuron are called a motor unit. Upon the generation of an action potential great enough to cause contraction, the neural impulse is transmitted to the myocyte through the neuromuscular junction and is propagated down the transverse tubules to the sarcoplasmic reticulum, causing Ca^{++} to be released. Ca^{++} binds to the troponin molecule, which causes a shift in tropomyosin position. This allows for exposure of the actin active sites and myosin-actin crossbridge formation, resulting in contraction.

Regulation of Skeletal Muscle Growth and Repair

The extracellular matrix (ECM) is a non-cellular structure that provides physical integrity and biomechanical/biochemical support for the surrounding cells. The ECM has two primary components denoted as the interstitial matrix

and the basement membrane (Koeppen & Stanton, 2010). These structures are largely made up of fibrous proteins such as collagen and elastin, which respectively provide rigidity and distensibility. The interstitial matrix serves as a compression support for the ECM, while the basement membrane is a fibrous sheet that separates the surrounding cells from the connective tissues. The ECM is a well-regulated structure that is synthesized and degraded to allow for proper wound healing, growth and fibrosis.

The extracellular matrix (ECM) of skeletal muscle is a very rigid structure that helps to support the high levels of mechanical stress that is placed on the muscle tissue. Much like the ECM's of other cell types, skeletal muscle ECM is largely comprised of collagen, with collagens I and III being predominant in the epi-, peri- and endomysium and collagen IV being predominant in the basement membrane of adult skeletal muscle (Gillies & Lieber, 2011; Light & Champion, 1984). Typical ECM proteins such as proteoglycans, glycoproteins and matrix remodeling enzymes can also be found in skeletal muscle along with several types of mononuclear cells. Fibroblasts are the predominant type of mononuclear cell in the skeletal muscle ECM and are largely responsible for producing the majority of the ECM components such as fibronectin, collagen and MMP's (Archile-Contreras, Mandell, & Purslow, 2010; Gatchalian, Schachner, & Sanes, 1989; Kuhl et al., 1984).

Although fibroblasts are the predominant mononuclear cell in skeletal muscle, macrophages are typically present in considerable number as well. Damaged muscle fibers attract monocytes to the site of injury through

chemotaxis. These monocytes switch to an anti-inflammatory profile before differentiating into macrophages. Upon phagocytosis of muscle cell debris, macrophages shift towards an anti-inflammatory profile, which causes them to release growth factors such as transforming growth factor β (TGF- β) and basic fibroblast growth factor (bFGF), resulting in stimulation of muscle satellite cell migration (Arnold et al., 2007; Robertson, Maley, Grounds, & Papadimitriou, 1993). Tissue injury causes significant increases in macrophage infiltration 2 days post injury and a substantially greater increase after 3 days. However, these levels begin to subside by day 6 (Pimorady-Esfahani, Grounds, & McMenamin, 1997). Research suggests that macrophages are rather crucial for skeletal muscle growth and repair. Inflammatory macrophages stimulate myogenic cell proliferation, while anti-inflammatory macrophages stimulate myogenic cell differentiation into myotubes (Arnold et al., 2007). Complete removal of macrophages after muscular injury hinders the repair process and decreases the diameter of regenerating muscle fibers.

Myostatin and Follistatin

Tissue growth and repair is tightly regulated by factors secreted by the skeletal muscle (myokines) as well. Myostatin, a member of the transforming growth factor β (TGF- β) superfamily, is a myokine that is well-known for its ability to inhibit muscular growth. It has been demonstrated that hindlimb unloading induced muscular atrophy in mice resulted in elevated myostatin levels that were apparent after 1 day in the gastrocnemius muscle (Carlson, Booth, &

Gordon, 1999). Reisz-Porszasz et al. demonstrate that myostatin over-expressing mice exhibit decreased bodyweight, muscle mass, myonuclear number and muscle fiber cross sectional area in the quadriceps, gastrocnemius and tibialis anterior muscles when compared to wild type (WT) controls (Reisz-Porszasz et al., 2003). Conversely, another study by Whittemore et al. demonstrates that inhibition of myostatin using an antibody blockade in C57 (WT) male and BALB/c female mice results in elevated quadriceps and gastrocnemius weights, increased total bodyweight and lean mass as well as increased grip strength (Whittemore et al., 2003). Research suggests that myostatin may inhibit muscle growth through suppression of satellite cell differentiation and myoblast proliferation (Joulia et al., 2003; Thomas et al., 2000; Wehling, Cai, & Tidball, 2000). Muscle fibers with higher expression of myostatin exhibit lower satellite cell numbers (Wehling et al., 2000). However, it appears that myostatin inhibits myoblast proliferation through halting myoblast cell cycle progression in the G0 (resting), G1 and G2 (growth) phases of the cell cycle, preventing myoblasts from turning into new myocytes (Joulia et al., 2003; Thomas et al., 2000).

While myostatin serves as a direct inhibitor of muscle growth by preventing differentiation of satellite cells and proliferation of myoblasts, follistatin (Fst), an autocrine glycoprotein, is a known inhibitor of members of the TGF- β superfamily, including myostatin. Lee et al. demonstrated that follistatin overexpressing mice exhibited dramatic increases in muscle mass that rivaled those of myostatin null mice (Lee & McPherron, 2001). It appears that myostatin elicits its inhibitory effects through binding of the activin receptor IIB (Act RIIb).

Follistatin not only directly binds to and inhibits myostatin, but also blocks myostatin signaling at the ActRIIB. Mice overexpressing a dominant negative form of the ActRIIB also displayed dramatic increases in weight and muscle mass. Similarly, Nakatani et al. utilized a mouse model that expresses a follistatin derived inhibitor (FSII) of myostatin that doesn't affect activin signaling. FSII expressing mice exhibited substantial increases in muscle mass and strength compared to WT mice (Nakatani et al., 2008). When crossbred with mdx mice (dystrophin knockout mice), which usually display severe muscular atrophy and weakness, these FSII/mdx mice have elevated strength and mass compared to mdx mice. In mdx mice, excessive macrophage infiltration into the skeletal muscle is a major factor that causes adverse skeletal muscle remodeling. The FSII/mdx mice also display a substantial decline in macrophage number in the quadriceps femoris compared to mdx mice (Nakatani et al., 2008). Another study from Lee et al. demonstrated that follistatin heterozygous knockout mice have a significant reduction in muscle size and a shift towards a more oxidative fiber type (Lee et al., 2010). These mice have reduced myocyte cross sectional areas, produce less twitch force and tetanic force and exhibit impaired muscle remodeling in response to injury.

Pathological Remodeling

During pathological conditions, the structure of skeletal muscle can become adversely remodeled, causing it to become fibrotic and excessively rigid, preventing proper function. Skeletal muscle fibrosis is apparent in diabetes,

immobilization and muscular dystrophy (Alexakis, Partridge, & Bou-Gharios, 2007; Berria et al., 2006; Duance, Stephens, Dunn, Bailey, & Dubowitz, 1980; Williams & Goldspink, 1984). Fibrotic muscle is characterized by increased collagen deposition due to elevated activity of fibroblasts, satellite cells, myofibers and inflammatory cells such as monocytes/macrophages. Following pathological or chronic tissue injury, myostatin directly stimulates fibroblast activity via activin receptor IIB, causing fibroblast deposition of fibrinogen and collagen (McCroskery et al., 2005). Furthermore, activated fibroblasts directly stimulate inflammatory macrophages through integrin receptor MAC-1, arousing their production of inflammatory factors such as interleukin 1- β (IL-1 β) and tumor necrosis factor- α (TNF- α) (Vidal et al., 2008). In pathological states related to chronic inflammation, processes such as these continue, causing skeletal muscle fibrosis and dysfunction.

It has been well established that mdx mice, a model that represents muscular dystrophy in humans, display a decrease in skeletal muscle mass, myocyte cross sectional area and muscular strength, as well as elevated macrophage infiltration and muscle fibrosis (Bogdanovich et al., 2002; Desguerre et al., 2012; Gutpell, Hrinivich, & Hoffman, 2015; Nakatani et al., 2008). Gutpell et al. demonstrate that 8 week old mdx/utrn +/- mice, which lack dystrophin and utrophin (another crucial protein in skeletal muscle structure), display extensive fibrosis in the gastrocnemius and diaphragm; while age matched mdx mice only displayed fibrosis in the diaphragm (Gutpell et al., 2015). Other studies suggest that myostatin inhibition significantly ameliorates muscular function in mdx mice,

producing elevated muscle weight, contractile force and cross sectional area, as well as attenuating macrophage infiltration into the skeletal muscle (Bogdanovich et al., 2002; Nakatani et al., 2008).

Homocysteine

Homocysteine Metabolism

Homocysteine (Hcy) is a sulfur containing, non-essential amino acid that is derived from dietary methionine. After absorption from the digestive tract, methionine is converted into S-adenosylmethionine (SAM) by ATP and methionine adenosyl triphosphate, leaving it with a transferrable methyl group. The methyl group is donated for normal cellular methylation requirements and becomes S-adenosyl-homocysteine (SAH). SAH hydrolase is an enzyme that reversibly hydrolyzes SAH, which produces homocysteine (Selhub, 1999).

Homocysteine is typically metabolized through two key processes known as trans-sulfuration and remethylation (See Figure 1). Through the trans-sulfuration process, homocysteine is irreversibly converted into cystathione via vitamin B6 dependent enzyme Cystathione Beta Synthase (CBS). Cystathione is then hydrolyzed by cystathione gamma-lyase into cysteine. This process occurs in the kidneys, liver, pancreas and the small intestines and allows for homocysteine molecules to be permanently removed from the body. However, through the remethylation pathway, homocysteine is remethylated back into methionine by methionine synthase (MS), which depends upon folate (vitamin

B12) as a cofactor. MS uses N-5-methyl tetrahydrofolate (methyl-THF) as a methyl donor to convert homocysteine into methionine, producing THF. THF is remethylated through a reaction catalyzed by methyl tetrahydrofolate reductase (MTHFR) back into methyl-THF. This process occurs in most tissues except for the liver and kidneys, where betaine serves as methyl donor through a reaction catalyzed by betaine-homocysteine-methyltransferase (BHMT) (Selhub, 1999).

Homocysteine Metabolism

Figure 1. Schematic representing the overview of homocysteine formation and metabolism.

Hyperhomocysteinemia

Homocysteine is present in healthy human plasma at a range of 5 to 15 μ M (Hillenbrand, Hillenbrand, Liewald, & Zimmermann, 2008).

Hyperhomocysteinemia (HHcy) is a condition where plasma Hcy levels become >15 μ M/L. HHcy has been implicated as a potential cause of numerous disease states such as hypertension, stroke, cardiovascular disease, and skeletal muscle dysfunction. HHcy is directly correlated with aortic stiffness, decreased renal function, endothelial dysfunction, and hypertension, with increases in homocysteine eliciting linear increases in the aforementioned pathologies, suggesting a causative effect (Bortolotto et al., 1999; Naess et al., 2013; Sutton-Tyrrell et al., 1997; Wiernicki et al., 2011; Zheng et al., 2013).

Hyperhomocysteinemia occurs when there is impairment in one or more of the metabolic processes used to remove homocysteine from the body. CBS or MTHFR mutations, low dietary folate, impaired renal clearance, or a high dietary intake of methionine are known causes of HHcy (Hayden & Tyagi, 2004; Kanwar et al., 1976; S. C. Tyagi et al., 2005; Veeranki & Tyagi, 2013). Several of the mechanisms through which HHcy may cause disease have been discovered. Hcy increases reactive oxygen species (ROS) production through the formation of mixed disulfides, protein homocysteinylation, and through auto-oxidation (Signorello et al., 2007; S. C. Tyagi et al., 2005). Homocysteine has been shown to cause dephosphorylation and uncoupling of endothelial nitric oxide synthase (eNOS), leading to a decrease in endothelial NO production (Tyagi et al., 2009). Decreased nitric oxide attenuates vascular responsiveness to wall shear caused

by changes in blood flow, preventing proper vaso-relaxation. Moreover, the increased wall stress associated with less pliable vessels elevates arterial pressures and decreases perfusion to the surrounding and distal tissues (Jacobson et al., 2007; Levy et al., 2008). This reduction in perfusion increases ROS production, starting a vicious cycle of ROS accumulation and tissue damage.

Signorello et al. demonstrated that type 2 diabetics have increased total plasma homocysteine levels when compared to control subjects (Signorello et al., 2007). It was found that the platelets of type 2 diabetic patients had significantly lower basal NO levels, decreased levels of reduced glutathione (an intracellular anti-oxidant), and increased ROS production when compared to those of control subjects. Hcy has also been shown to inactivate peroxisome proliferator activated receptor – α and – γ (PPAR- α , PPAR- γ), which are pro-antioxidants that have been shown to promote synthesis of superoxide dismutase (SOD) and decrease oxidative stress (Mujumdar, Tummalapalli, Aru, & Tyagi, 2002). This decreased activity of PPAR by Hcy may escalate constrictive vascular remodeling.

Hyperhomocysteinemia and Skeletal Muscle

Although common muscular disorders, such as muscular dystrophy, sarcopenia and immobilization, have been extensively researched, there is not a lot of data available on the effects of HHcy on skeletal muscle function. Swart et al. demonstrated that in elderly individuals (mean = 75.6 years of age), plasma

Hcy levels had a strong inverse correlation with grip strength and functional capabilities. High Hcy levels resulted in poor grip strength in men and lower functional ability, with functional abilities including walking, climbing stairs and rising from a chair (Swart et al., 2013). In cystathione beta synthase deficient mice (CBS+/-), a model for mild to moderate HHcy, It was demonstrated that 28 days after induction of hind limb ischemia CBS+/- mice displayed blunted hind limb perfusion and lower collateral blood vessel development when compared to WT mice (Veeranki, Givvimani, Pushpakumar, & Tyagi, 2014). CBS+/- mice also demonstrated blunted vascular endothelial growth factor (VEGF), hypoxia inducible factor (HIF1- α) and peroxisome proliferator activated receptor coactivator 1- α (PGC1- α) after 28 days of ischemic recovery, when compared to control. Another study by Veeranki et al. demonstrates that CBS+/- skeletal muscle is less fatigue resistant, produces less contractile force and has lower muscle ATP levels compared to C57 wild type mice (S. Veeranki et al., 2015). It was discovered that CBS+/- skeletal muscle has lower dystrophin and mitochondrial transcription factor A (mtTFA) when compared to controls (See Figures 2 and 3 below). Although these studies demonstrate some of the effects of HHcy on skeletal muscle, more data is needed to determine the mechanism by which HHcy causes skeletal muscle impairment.

Figure 2: Maximal force production is decreased in the soleus and EDL of CBS +/- mice, possibly due to decreased skeletal muscle ATP generation.

The above data are reproduced from a 2015 article by Veeranki et al., (S. Veeranki et al., 2015). The above data demonstrate that maximal force generation is reduced in the A)EDL and B)soleus of CBS +/- mice. This is likely due to an observed decrease in C) skeletal muscle ATP production.

Figure 3: CBS+/- mice have lower skeletal muscle expression of Dystrophin, mtTFA and NRF-1; this is reversed with exercise. The above data are reproduced from a 2015 article by Veeranki et al., (S. Veeranki et al., 2015). These data indicate that A) dystrophin, mtTFA and B) nuclear respiratory factor (NRF-1), a transcriptional regulator of mtTFA, are significantly reduced in CBS +/- mice compared to C57. Exercise increases mtTFA and NRF-1 levels.

Matrix Metalloproteinase-9

Matrix Metalloproteinases (MMP's) are largely responsible for the proper degradation of the ECM in healthy individuals. MMP's are zinc and calcium dependent endoproteinases that are secreted by many cell types such as macrophages, fibroblasts, vascular smooth muscle cells and myocytes. Matrix Metalloproteinase-9 (MMP-9) is classified as a gelatinase that has the ability to degrade components of the extracellular matrix (ECM), including collagen type IV and elastin (the main elastic component of the arterial wall). MMP-9 is stimulated through activation of extracellular matrix metalloproteinase inducer (EMMPRIN) via p38 MAPK stimulation (Huang et al., 2011; Reddy et al., 2010), as well as through extracellular signal-regulated kinase (ERK)/ nuclear factor- κ B (NF- κ B) dependent activator protein (AP-1) activation (Fan, Meng, Wang, Cao, & Wang, 2011; Seung Jin Lee et al., 2012; Moshal et al., 2006; Reddy et al., 2010; Neetu Tyagi et al., 2009). In healthy muscle tissues, MMP-9 is known for playing a role in the repair and remodeling of the tissue as well as angiogenesis (Bellafiore et al., 2013; Chen & Li, 2009; Kherif et al., 1999; Lewis, Tippet, Sinanan, Morgan, & Hunt, 2000; Mackey, Donnelly, Turpeenniemi-Hujanen, & Roper, 2004; Zimowska, Brzoska, Swierczynska, Streminska, & Moraczewski, 2008). It has been localized to cardiomyocytes, around skeletal muscle fibers, capillaries and within the skeletal muscle ECM (Bellafiore et al., 2013; Rullman et al., 2009). Research has demonstrated that MMP-9 is crucial for the onset of angiogenesis (Bellafiore et al., 2013) and for maintaining proper compliance and distensibility of arteries (Flamant et al., 2007). During growth and repair of skeletal muscle,

MMP-9 has been shown to stimulate satellite cell migration and differentiation through degradation of the ECM basement membrane, assisting with their mobility into the muscle (Chen & Li, 2009; Kherif et al., 1999; Lewis et al., 2000; Zimowska et al., 2008).

MMP-9 and Disease

Excessive inflammation and oxidative stress can lead to abnormal activation of MMP-9, causing excessive degradation of the ECM, abnormal vascular remodeling and endothelial dysfunction, which are associated with hypertension and arterial stiffness (Onal et al., 2009; Wallace et al., 2005). Wallace et al. demonstrate that patients with isolated systolic hypertension had significantly elevated MMP-9 levels and higher pulse wave velocities (PWV, a measure of arterial stiffness) when compared to age-matched controls (Wallace et al., 2005). It was determined that MMP-9 levels correlated linearly with PWV, suggesting that MMP-9 may be, at least in part, a cause of arterial stiffening and hypertension.

Tyagi et al. demonstrate that mitochondrial induced oxidative stress activation of MMP-9 leads to degradation of the gap junction protein connexin-43 (Cx-43) in the myocardium (N. Tyagi, Vacek, Givvimani, Sen, & Tyagi, 2010). Degradation of Cx-43 elicits fibrosis and ventricular dysfunction. Another study by Onal et al. confirms the finding that MMP-9 levels are higher in individuals with hypertension and that antihypertensive treatment lowers MMP-9 to control group levels (Onal et al., 2009). Furthermore, MMP-9 is induced by EMMPRIN in the

atherosclerotic carotid lesion of advanced atherosclerotic plaque, leading to abnormal ECM remodeling and atheroma instability (Yoon et al., 2005).

MMP-9 and Skeletal Muscle

Although there has been extensive research on the effects of MMP-9 in cardiovascular disease and vascular remodeling, the data on the effects of abnormal MMP-9 expression in skeletal muscle diseases is relatively limited. With MMP-9 playing a large role in collagen/elastin degradation in the ECM of skeletal muscle tissues, it would suggest that MMP-9 hyperactivity could create pathological conditions. In Duchenne's muscular dystrophy (DMD), which is characterized by skeletal muscle weakness and atrophy, it was determined that MMP-9 levels are significantly elevated in serum samples of mdx mice and DMD patients when compared to their healthy counterparts (Nadarajah et al., 2011). Similarly, people and animals infected with immunodeficiency viruses suffer from exacerbated muscle wasting. In rhesus macaques infected with the simian immunodeficiency virus, chronic binge alcohol administration leads to significant increases in MMP-9 mRNA expression in skeletal muscle and was associated with ECM remodeling and fibrosis (Dodd et al., 2014).

MMP-9 and Hyperhomocysteinemia

Hyperhomocysteinemia is a well-known stimulus for MMP-9 activity (Lee et al., 2012; Moshal et al., 2006; Tyagi et al., 2009) and strongly correlates with its activity in vascular disease (Wiernicki et al., 2011). Hcy stimulates MMP-9

production in several cell types including ventricular endothelial cells, brain endothelial cells and macrophages. Studies suggest that Hcy largely upregulates MMP-9 expression in these cell lines through an oxidative stress induced ERK/AP-1 pathway (Lee et al., 2012; Moshal et al., 2006; Tyagi et al., 2009). Tyagi et al. determined that HHcy also induces MMP-9 activation through ERK, leading to degradation of the extracellular matrix (Tyagi et al., 2009). It was shown that homocysteine acts as an antagonist of the gamma aminobutyric acid –A (GABA-A) receptor, leading to increased ROS production, increased MMP-9 activity, and decreased NO production through the uncoupling of eNOS. Pharmacological stimulation of the GABA-A receptor attenuates Hcy induction of MMP-9. The GABA-A receptor controls several antioxidative enzymes, such as thioredoxin and NADPH oxidase-4, indicating enhanced ROS production acts as the MMP-9 stimulus. Lee et al. determined that Hcy induction of MMP-9 also occurs through a separate Akt pathway in murine macrophages (Seung Jin Lee et al., 2012).

EMMPRIN induction of MMP-9

Extracellular matrix metalloproteinase inducer, or EMMPRIN, is a membrane bound protein found in most cell types. It is known for mediating cellular signaling cascades, particularly those stimulating MMP's (Venkatesan et al., 2010). Elevated EMMPRIN expression is associated with ROS production (Siwik et al., 2008) and numerous disease states such as atherosclerosis (Major, Liang, Lu, Rosebury, & Bocan, 2002), rheumatoid arthritis (Zhu et al., 2005) and

cancer (Guo, Li, Zucker, & Toole, 2000). Extensive research has demonstrated that EMMPRIN is a potent inducer of MMP-9 (Fan et al., 2011; Reddy et al., 2010; Tarin et al., 2011; Yoon et al., 2005). Studies suggest that EMMPRIN is stimulated by several inflammatory molecules including the inflammatory cytokines such as interleukin-18 and visfatin as well as cyclophilin A, a potent ROS inducer (Fan et al., 2011; Reddy et al., 2010; Yuan, Ge, & He, 2010). The exact pathways for EMMPRIN stimulation are still largely undetermined, however, it appears that these molecules stimulate EMMPRIN through a cGMP, p38-ERK 1/2-NF- κ B signaling pathway (Fan et al., 2011; Huang et al., 2011; Tarin et al., 2011). Interestingly, it appears that EMMPRIN utilizes the same p38/ERK/NF- κ B pathway to stimulate other molecules such as MMP-9 (Reddy et al., 2010; Yuan et al., 2010). EMMPRIN and MMP-9 co-expression are found prevalently in atherosclerotic plaques/atheromas and ischemia/reperfusion injury to cardiac tissue (Major et al., 2002; Tarin et al., 2011; Yoon et al., 2005).

Macrophages

Macrophages are mononuclear white blood cells that are derived from differentiating monocytes in tissues. They are used both in adaptive and innate immune responses and are phagocytic by nature, meaning they engulf debris and pathogens for disposal. Macrophages stimulate the immune system by signaling other immune cells such as lymphocytes and promote inflammation through the release of pro-inflammatory cytokines, ROS and NO, which are used as an attack mechanism against invading pathogens. After phagocytosis of pathogen, macrophages present the antigen to a corresponding helper T cell, which then signals for the production of antibodies against the antigen, helping with the body's adaptive immunity. Although macrophages are necessary for adequate immune response, they have been suggested to exacerbate a number of diseases such as obesity, type II diabetes, atherosclerosis, and cancer (Mills, 2012; Sica & Bronte, 2007; Sica & Mantovani, 2012; Tavakoli & Asmis, 2012).

M1 vs M2 Macrophages

The phenotype and function of macrophages is controlled by environmental signals. Two polarized phenotypes of macrophages are denoted as M1 or M2. The M1 phenotype is the destructive, “classically activated” subset, while the M2 is called the “alternatively activated” subset, associated with growth and repair (Mills, 2012; Sica & Mantovani, 2012). The M1 phenotype is stimulated by Interleukin-12 (IL-12), lipopolysaccharide and interferon- γ and can be identified by high expression of the cell surface marker CD40 (Aron-

Wisniewsky et al., 2009; Munder, Mallo, Eichmann, & Modolell, 1998; Sica & Mantovani, 2012). M2 macrophages are known to be stimulated by interleukin-10 (IL-10) and interleukin-4 (IL-4) and can be identified by the cell surface markers CD163 and CD206 (Aron-Wisniewsky et al., 2009; Fiorentino, Zlotnik, Mosmann, Howard, & O'Garra, 1991; Lang, Patel, Morris, Rutschman, & Murray, 2002; Sica & Mantovani, 2012).

Macrophages are recruited to a specific area through chemical signaling known as chemotaxis. When the resulting phenotype of the signaled macrophage is M1, the macrophage attacks pathogens with a barrage of pro-inflammatory factors. The M1 macrophages will release pro-inflammatory cytokines, ROS, and NO through increased production of inducible nitric oxide synthase (iNOS) (Leibovich et al., 1987; Mills, 2012; Sica & Mantovani, 2012; Weisser, McLarren, Kuroda, & Sly, 2013). When signaled to damaged endothelium or areas of arterial damage, the pro-inflammatory action elicited by M1 macrophages is thought to initiate or progress the damage that causes vascular pathologies such as atherosclerosis.

Conversely, the M2 phenotype is known to help promote survival and angiogenesis through the release of anti-inflammatory cytokines and growth factors. M2 macrophages have been shown to release IL-10, an inhibitor of pro-inflammatory cytokines, PPAR- γ , which is a pro-antioxidant that improves insulin sensitivity, and VEGF, which is associated with angiogenesis, as well as other proliferative factors (Fiorentino et al., 1991; Lang et al., 2002; Mills, 2012; Odegaard et al., 2007; Sica & Mantovani, 2012; Zeyda et al., 2007).

M1 and M2 Macrophages in Injury and Disease

Kigerl et al. were able to demonstrate the difference between the M1 and M2 phenotype in an injured mouse spinal cord (Kigerl et al., 2009). Their data suggest that M1 macrophages rapidly respond at sites of spinal cord damage and produce a neurotoxic effect, which profoundly overpowers the M2 response. In contrast, it is shown that induction of the M2 phenotype in these macrophages actually elicits axonal growth for neural regeneration.

Obesity displays a low level of chronic inflammation that is thought to be associated with the development of associated diseases, such as insulin resistance. Aron-Wisnewsky et al. demonstrate that M1 macrophages are significantly increased in the adipose tissue of obese women (Aron-Wisnewsky et al., 2009). After 3 months of gastric bypass surgery, it was found that the ratio of M1/M2 macrophages was 2-fold lower than pre-surgery, suggesting a switch to a less inflammatory profile.

El Hadri et al. found that when stimulated with thioredoxin-1, an anti-inflammatory protein, macrophages are shifted to an M2 phenotype and this switch antagonizes atherosclerosis (El Hadri et al., 2012). In contrast, despite the proliferative, anti-inflammatory nature of M2 macrophages, it has been discovered that M2 macrophages may also be associated with increased atherogenesis. Oh et al. demonstrated that M2 macrophages are responsible for foam cell formation and deposition into the intima of the vascular wall, promoting arterial plaque formation (Oh et al., 2012). IL-4 and IL-10 stimulation of macrophages promoted ER stress that generated PPAR- γ and JNK activation,

leading to an M2 phenotype. When the M2 macrophages were exposed to oxidized low density lipoprotein, foam cell formation occurred because of CD36 and scavenger receptor-A1 (SR-A1) induction. This transformation of M2 macrophages into foam cells could play a major role in the formation and progression of atherosclerotic plaque.

Macrophages and Hyperhomocysteinemia

Interestingly, homocysteine shares a causative link with the atherogenic actions of M1 and M2 macrophages. Research suggests that that HHcy induces VEGF and monocyte chemoattractant protein-1 (MCP-1, a potent chemotactic agent), expression via an NF- κ B dependent pathway in THP-1 human macrophages, which are both associated with vascular disease progression (Wang et al., 2001). This study demonstrates HHcy stimulates macrophage migration into the arterial intima through a 4.8 fold induction of MCP-1. Other studies also demonstrate that Hcy upregulates VEGF production in macrophages via NF- κ B activation (Kiriakidis et al., 2003; Maeda, Yamamoto, Fujio, & Azuma, 2003). More recently, a study by Lee et al. suggested that Hcy treatment resulted in elevated MMP-9 production in the J774A.1 murine macrophage cell line (Lee et al., 2012). 12 hours of 300 μ M Hcy treatment resulted in significantly elevated MMP-9 protein expression and activity. Further analysis revealed that this induction occurred through ERK and Akt pathways.

Macrophages in Skeletal Muscle

Macrophages are typically present in substantial quantity in the skeletal muscle, largely in the epimysium and perimysium (Chazaud et al., 2009). One major role of macrophages in the skeletal muscle is to assist with tissue growth and repair. Damaged muscle fibers attract monocytes to the site of injury through chemotaxis, where the monocytes differentiate into macrophages. Upon phagocytosis of muscle cell debris, macrophages shift towards an anti-inflammatory profile, which causes them to release growth factors such as TGF- β and basic fibroblast growth factor (bFGF), resulting in stimulation of muscle satellite cell migration (Arnold et al., 2007; Robertson et al., 1993). The macrophage shift towards an anti-inflammatory or M2 profile seems to occur between 1 to 3 days post injury (Chazaud et al., 2009). Tissue injury causes significant increases in macrophage infiltration 2 days post injury and a substantially greater increase after 3 days. However, these levels begin to subside by day 6 (Pimorady-Esfahani et al., 1997).

Research suggests that macrophages are rather crucial for skeletal muscle growth and repair. If monocyte recruitment to the site of damaged skeletal muscle is interrupted within the first 24 hours post-injury, the repair process is completely attenuated. Inflammatory macrophages stimulate myogenic cell proliferation, while anti-inflammatory macrophages stimulate myogenic cell differentiation into myotubes (Arnold et al., 2007). Complete removal of macrophages after muscular injury also hinders the repair process

and decreases the diameter of regenerating muscle fibers (Arnold et al., 2007; Chazaud et al., 2009).

As discussed earlier, during pathological conditions, macrophage infiltration into the skeletal muscle can be heavily increased and promote a potent inflammatory profile. This chronic state of inflammation elicits adverse muscle remodeling through tissue fibrosis and muscular atrophy.

Exercise Training

It has been well established that regular exercise is associated with improvements in overall physical fitness, maintenance of health, a decreased risk of many disease states, and more recently, alleviation of many disease pathologies (Fiuza-Luces, Garatachea, Berger, & Lucia, 2013). Despite this common knowledge, technological advancements associated with modern day living have caused dramatic increases in sedentary lifestyle. Approximately 43% of American adults and 31% of adults worldwide are considered to be physically inactive (Hallal et al., 2012). This lack of physical exercise has been associated with elevated cardiovascular disease mortality risk and is estimated to be attributable for 16% of all-cause death (Blair, 2009). Furthermore, decreased physical activity has also been associated with the development of hypertension and obesity (Tremblay, Esliger, Copeland, Barnes, & Bassett, 2008), which are known to cause a wide variety of other disease states.

Supporting the health promoting advantages of regular exercise, a review by Fiuza-Luces et al. suggests that regular exercise has physiological health benefits that are equivalent to a multi-drug (blood thinner, anti-diabetic, anti-hypertensive, anti-lipid/cholesterolemic) regimen (Fiuza-Luces et al., 2013). When compared to the benefits of this multiple drug treatment program, exercise is as capable, if not more proficient, at improving lipid profile, blood pressure, diabetes prevention, and reduction of cardiovascular events. Most of these benefits are derived from chemicals released by contracting skeletal muscle (myokines), such as IL-6, which increases the production of anti-inflammatory cytokines, angiogenic factors, and follistatin, which improves muscle growth and possibly insulin sensitivity through inhibition of myostatin (Pedersen, Akerstrom, Nielsen, & Fischer, 2007).

Acute exercise has been shown to elevate NO production through a 37% increase in nitric oxide synthase (NOS) in rat gastrocnemius muscle (Roberts, Barnard, Jasman, & Balon, 1999). Through adaptation, regular exercise improves endothelial function, at least in part, due to elevated endothelial nitric oxide synthase (eNOS) mRNA expression, allowing for enhanced NO production and vasoreactivity (Fogarty et al., 2004; Griffin, Woodman, Price, Laughlin, & Parker, 2001; Hambrecht et al., 2000). Improved stem cell production and release also contributes to the benefits derived from exercise (Macaluso & Myburgh, 2012). Endothelial progenitor cells stimulated by exercise have been shown to migrate to the damaged endothelium to initiate repair or angiogenesis, improving vascular function (Crosby et al., 2000; Moreno, Sanz, & Fuster, 2009).

It has also been revealed that exercise improves cellular responsiveness to stress through improved metabolic efficiency and elevated anti-oxidative capacity, resulting in decreased free radicals and mitigation of ROS induced pathologies (Gomez-Cabrera et al., 2005; Jackson, 2005; Khassaf et al., 2001; McArdle, Pattwell, Vasilaki, Griffiths, & Jackson, 2001; Radak, Chung, & Goto, 2008).

Exercise and Homocysteine

It has been demonstrated that exercise is capable of lowering Hcy levels, suggesting that exercise can potentially negate the adverse effects of HHcy on different organs (Hrncic et al., 2013; König et al., 2003; Neuman et al., 2013; Randeva et al., 2002; Vincent et al., 2006). Neuman et al. stated that exercise was capable of preventing increases in plasma Hcy concentration that were produced by 7 weeks of a folate restricted diet in mouse models (Neuman et al., 2013). The low folate diet group that was not exercised displayed significant increases in Hcy levels when compared to non-exercise controls and controls receiving exercise. It was demonstrated that this prevention of hyperhomocysteinemia was associated with a 2 fold increase in renal betaine-homocysteine S-methyltransferase (BHMT) activity. BHMT is a folate independent methyltransferase that remethylates Hcy into methionine by utilizing a methyl group from betaine. Another study revealed the ability of exercise to prevent increases in lipid peroxidation and decreases in superoxide dismutase and catalase activity by Hcy in mice (Hrncic et al., 2013). Human studies have

also discovered that exercise lowers Hcy levels in overweight and obese adults as well as in women with polycystic ovarian syndrome (Randeva et al., 2002; Vincent et al., 2006).

Exercise and MMP-9

Despite an abundance of research on MMP-9 and its association with vascular complications, the effects of regular exercise on MMP-9 are rather unclear. After single acute bouts of exercise, it is shown that MMP-9 activity is elevated around the capillaries and cardiomyocytes (Bellafore et al., 2013; Rullman et al., 2009). Increased serum levels of MMP-9 have been reported after 8 days of a single exercise bout and MMP-9 remains elevated even after 15 days of training (Bellafore et al., 2013; Mackey et al., 2004; Rullman et al., 2009). This could suggest that MMP-9 is being utilized to help promote exercise induced angiogenesis. Interestingly, although MMP-9 activity is increased after a single exercise bout, it has been revealed that there is no change in enzyme activity after extended training regimens, therefore, it is possible that MMP-9 activity may only be utilized in the initial phases of new capillary growth (Rullman et al., 2009).

Exercise and Macrophages

It is known that acute bouts of exercise stimulate the phagocytic activity of macrophages (De la Fuente, Martin, & Ortega, 1990; Ferrandez & De la Fuente, 1999). This makes sense since macrophages play a crucial role in phagocytosis of muscle debris and the muscular repair process. Regular exercise appears to

increase phagocytic activity of macrophages as well, which is more exaggerated in the elderly, whom typically have a blunted macrophage response to pathogens (Ferrandez & De la Fuente, 1999). This could be an important mechanism through which exercise improves immune responsiveness. Although the effect of exercise on macrophage activity has been well researched, there seems to be little information available regarding the effects of regular exercise on M1 vs M2 macrophage phenotype differentiation. One study suggests that mice being fed a high fat diet display a greater ratio of adipose tissue M1/M2 macrophages compared to normal diet controls (Kawanishi, Yano, Yokogawa, & Suzuki, 2010). After 16 weeks of exercise training, not only was the increased ratio of M1/M2 macrophages attenuated in the high fat diet group, but the expression level of M2 macrophages was markedly increased. This was accompanied by a decrease in inflammatory cytokines. Since this is the only study involving exercise and M1/M2 macrophages, it is clear that more research is needed regarding M1 vs M2 macrophage modification by exercise.

CHAPTER III

HYPOTHESIS AND SPECIFIC AIMS

Key Objective

The objective of this study was to determine the effects of hyperhomocysteinemia on skeletal muscle dysfunction and investigate if exercise can attenuate these effects.

Hypothesis

Hyperhomocysteinemia promotes adverse remodeling of skeletal muscle through induction of inflammatory mechanisms and exercise will mitigate this inflammatory state (See Figure 4).

Specific Aims

Specific Aim 1: To investigate if HHcy impairs skeletal muscle perfusion and function and determine if exercise mitigates this effect.

Specific Aim 2: To investigate changes to the inflammatory state of the skeletal muscle due to HHcy and determine if exercise can reverse this effect.

Specific aims 1 and 2 will be discussed in Chapter IV.

Specific Aim 3: To investigate changes in macrophage phenotype and skeletal muscle infiltration due to HHcy and determine if exercise can mitigate this effect.

Specific aim 3 will be discussed in Chapter V.

Figure 4: Schematic Representation of Overall Hypothesis

CHAPTER IV

SKELETAL MUSCLE DYSFUNCTION DUE TO HYPERHOMOCYSTEINEMIA

Introduction

Hyperhomocysteinemia (HHcy), having plasma homocysteine levels $>15\mu\text{M}$, is a significant risk factor associated with hypertension, atherosclerosis, abdominal aortic aneurysms and diabetes (Bortolotto et al., 1999; Signorello et al., 2007; Sutton-Tyrrell et al., 1997; Wiernicki et al., 2011; Zheng et al., 2013). HHcy can develop due to high dietary methionine, insufficient dietary folate, or genetic defects in enzymes that metabolize homocysteine, such as cystathione beta synthase or methyl-tetrahydrofolate reductase. People and animals with HHcy display pathological inflammation and remodeling of their cardiac and vascular smooth muscle (Bortolotto et al., 1999; Mujumdar et al., 2002; Shai et al., 2004; Signorello et al., 2007; Zheng et al., 2013). Muscular remodeling involves degradation and regeneration of fibrous extracellular matrix (ECM) proteins such as collagen, elastin and fibronectin. Pathological remodeling can occur during states of chronic inflammation and is characterized by excessive collagen deposition and depletion of elastin, causing the muscle to become fibrotic, unstable and rigid (Gillies and Lieber, 2011).

Homocysteine is known to induce several inflammatory factors such as reactive oxygen species (ROS), matrix metalloproteinase-9 and stimulation of macrophage migration through chemotaxis (Lee et al., 2012; Tyagi et al., 2009; Wang et al., 2001). MMP-9 is an endoproteinase that degrades collagen within the ECM. Its ability to degrade the extracellular matrix has been implicated in cardiovascular remodeling (Spinale et al., 2000; Tyagi et al., 2010). It is strongly associated with heart failure, hypertension and arterial stiffness and positively correlates with Hcy levels (Onal et al., 2009; Spinale et al., 2000; Wiernicki et al., 2011; Yasmin et al., 2005). Macrophages are required for the growth and repair of damaged muscle tissue, but chronic inflammation can cause macrophages to become hyperactive and destructive, leading to fibrosis of tissue. Hcy has been shown to stimulate macrophage migration, indicating that HHcy could promote remodeling through macrophage activation as well (Wang et al., 2001).

Limb immobilization, diabetes and muscular dystrophy all display pathological remodeling through excessive fibrosis of the skeletal muscle (Alexakis et al., 2007; Berria et al., 2006; Duance et al., 1980; Williams & Goldspink, 1984). Macrophages and MMP-9 are both present in the skeletal muscle for normal growth and repair, but the inflammation induced by HHcy may cause adverse skeletal muscle remodeling through amelioration of their activity. Skeletal muscle is a very metabolically active tissue and therefore requires a rich blood supply for proper perfusion and function (Holloszy & Coyle, 1984; Saltin, Radegran, Koskolou, & Roach, 1998). A lack of perfusion reduces clearance of metabolic oxidative byproducts, which would lead to inflammation. With HHcy

impairing blood flow through vascular dysfunction, it is very plausible that HHcy could promote or exacerbate pathological remodeling of skeletal muscle as well.

Individuals with HHcy produce lower maximal grip strength and impaired functional capabilities when compared to healthy counterparts (Swart et al., 2013). In CBS+/- mice, a model for HHcy, it is demonstrated that HHcy impairs hindlimb reperfusion angiogenesis after ischemia caused by femoral artery ligation (Veeranki et al., 2014). Another study from Veeranki et al. demonstrates that the skeletal muscle of CBS+/- mice is more fatigable and produces less contractile force than wild type mice. It was determined that the skeletal muscle has blunted ATP production, which may be the cause of the fatigability (Veeranki et al., 2015). The CBS+/- muscle also has decreased dystrophin and mitochondria transcription factor A (mtTFA) protein expression compared to wild type, indicating that the muscle is less functional and has lower mitochondrial activity. There was also an accompanied increase in mir-31 and mir-494, which respectively inhibit dystrophin and mtTFA. Additionally, there was a significant decrease in nuclear respiratory factor-1 (NRF-1) expression, which is a transcriptional promoter of mtTFA. A 4 week swimming protocol was administered to see if these effects could be attenuated. With the exception of dystrophin, all of the molecular changes observed in CBS+/- were reversed with exercise. With HHcy clearly causing functional and molecular changes to skeletal muscle, we decided to further investigate what changes HHcy is inducing in the skeletal muscle tissue. It is our hypothesis that HHcy impairs perfusion and

promotes pathological remodeling in skeletal muscle by inducing chronic inflammation. We believe that exercise will mitigate these effects.

Methods

Animals and Animal Care

C57BL/6J (WT), CBS+/- (B6129P2), FVB (FVB/NJ) and MMP-9-/- (FVB.Cg-Mmp9^{tm1Tvu}/J) were obtained from Jackson Laboratories. CBS+/- mice are heterozygous knockout mice that display HHcy and are based on the C57 WT model. MMP-9 -/- mice are based on the FVB (WT) model and are homozygous knockouts for MMP-9. CBS/MMP-9 double KO mice were bred in our animal facilities through mating of MMP-9-/- and CBS+/- mice. Only CBS/MMP-9 mice that were genotyped as being heterozygous knockouts for CBS and MMP-9 homozygous knockouts were used for this study. Animals were kept in our laboratories animal facility which exposes them to 12 hour of light and 12 hours of dark daily. They were fed normal chow and had free access to a fresh water supply. For the purpose of harvesting tissues, animals were fully anesthetized using tri-bromoethanol and euthanized via exsanguination. These methodologies are both considered humane and appropriate procedures by the University's IACUC. Gastrocnemius muscles were harvested for analysis and snap frozen in liquid nitrogen for western blotting and PCR analysis or cryopreserved in Peel-A-Way disposable tissue molds (Polysciences,

Warrington, PA) containing tissue freezing medium (Triangle Biomedical Sciences; Durham, NC) by freezing in liquid nitrogen for cryo-sectioning.

Exercise Protocol

All 5 mouse breeds had a control group that received no exercise training for 6 weeks and a group that was exercised on a 6 lane treadmill (Columbus Instruments; Columbus, OH) for 6 weeks. The treadmill protocol was personally designed while following mouse exercise guidelines as outlined in the “Resource Book for the Design of Animal Exercise Protocols,” which was published by the American Physiological Society in February of 2006 (Kregel et al., 2006). Prior to the start of exercise, mice were placed in the lanes of the treadmill daily for 5 days for 20 minutes per day with no belt movement to allow them to acclimate to the environment. Mice were exercised 5 days per week for 6 weeks with increasing intensity each day. The first two weeks were low intensity to allow for proper acclimation to a moving belt on the treadmill. The protocol consisted of a standard training phase for 20 minutes followed by a high-intensity sprint phase to induce muscular hypertrophy. Before the start of the study, it was determined that most mouse models had a very difficult time running at speeds greater than 20 m/min for more than a very short duration. To ensure that the protocol finished at a high intensity, a final training speed of 17 m/min (85% max speed at baseline) with a final sprint phase speed of 20 m/min (100% of max speed at baseline) were utilized. On the first day of exercise training, the mice began the training phase at 5 m/min for 20 minutes (or 100 m in 20 min) and a 15 m/min

sprint speed for 10 meters. The training phase increased by 0.5 m/min every day for the duration of the protocol until it reached 17 m/min for 20 minutes (or 340 meters in 20 min) during the final week, where the speed was held constant for the final 5 training sessions. The sprint phase distance at 15 m/min increased by 5 meters every training session until the half way point where a combination of 15 m/min and 20 m/min training speeds were utilized for 11 training sessions, followed by 10 training sessions at 20 m/min for increased distance. See figure 5 and table 1 below for clarification.

Figure 5. Line graph representation of the exercise protocol. Red indicates the distance run in 20 minutes for the standard training part of the protocol. Green represents the distance run at 15 m/min for the sprint phase, while blue represents the distance run at 20 m/min.

Day of		Speed For 20		Distance at		Distance at
Training		Mins (m/min)		15 m/min		20 m/min
1		5		10		0
2		5.5		15		0
3		6		20		0
4		6.5		25		0
5		7		30		0
6		7.5		35		0
7		8		40		0
8		8.5		45		0
9		9		50		0
10		9.5		50		5
11		10		50		10
12		10.5		50		15
13		11		50		20
14		11.5		50		25
15		12		50		30
16		12.5		50		35
17		13		50		40
18		13.5		50		45
19		14		50		50
20		14.5		50		50
21		15		0		80
22		15.5		0		85
23		16		0		90
24		16.5		0		95
25		17		0		100
26		17		0		100
27		17		0		100
28		17		0		100
29		17		0		100
30		17		0		100

Table 1. Table layout of the exercise protocol.

Blood Pressure Measurements

Blood pressure data was collected before the start of exercise (or control) and again two days following the completion of exercise training (or 6 weeks of control). Measurements were taken using a common, non-invasive tail cuff system (CODA; Kent Scientific: Torrington, CT). Animals were physically restrained using polymer harnesses designed for use with this system. The animals were placed on a warming platform for 10 minutes at 37°C to allow them to acclimate. Mean blood pressure measurements were recorded and used for this study.

Antibodies and reagents

Antibodies for homocysteine, myostatin and MMP-9 were purchased from Abcam (Cambridge, MA), while antibodies for VEGF, IL-1 β , PPAR- γ , EMMPRIN, iNOS and all secondary antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). The (glyceraldehyde 3-phosphate dehydrogenase) GAPDH antibody was purchased from EMD Millipore (Billerica, MA). All PCR primers were purchased from Invitrogen (Carlsbad, CA).

Plasma Homocysteine Measurement

To measure the differences in levels of homocysteine and homocysteinylated proteins between groups, blood samples were collected

through the vena cava of anesthetized animals. Sodium citrate was used to coat the interior of the syringe prior to blood collection to prevent coagulation. Blood samples were centrifuged at 6000Xg for 10 minutes and plasma (supernatant) was carefully pipetted into another tube. Plasma samples were stored at -80°C until needed. A dot blot was utilized to measure differences in plasma Hcy levels. Equal volumes of plasma from each sample were blotted onto a nitrocellulose membrane and allowed to dry for 1 hour. The membrane was placed in a 3% BSA/0.1% Tween 20 solution diluted in tris-buffered saline (TBS) at room temperature for 1 hour. Anti-Homocysteine rabbit polyclonal antibody was diluted 1:5000 in TBST solution and added to membrane for incubation overnight at 4°C. The membrane was then washed with TBST 3 times for 5 minutes per wash on a rocker at room temperature. Anti-rabbit secondary antibody was diluted 1:10,000 in TBST and added to membrane for 1 hour at room temperature. The dot blot was then imaged and developed using chemiluminescent substrate and analyzed using BioRad ImageLab software (Hercules, CA).

Laser Doppler Imaging

To measure the blood perfusion of the femoral artery, after receiving anesthesia, the mice were secured in the supine position and the femoral artery of the left hind limb was surgically exposed. Femoral artery perfusion was measured using a laser Doppler imager (MoorLDI; Moor Instruments: Devon,

UK) for a period of 2 minutes. The mean perfusion over the 2 minute period was calculated and used for analysis.

Ultrasonography

Ultrasonography was performed before the start of exercise (or control) and the day following completion of exercise training (or 6 weeks of control). While under isoflurane anesthesia, mice were placed on a warming platform at 37°C and a commercial hair removal chemical was used to remove all fur from the hind limb. Imaging of the femoral artery was performed using a Vevo 2100 ultrasound Doppler (Visual Sonics, Toronto, ON, Canada). The femoral artery was imaged using a MS550D (22-55 mHz) transducer. The B-Mode imaging program was used to obtain clear cross sectional images of the femoral artery were obtained to measure the lumen diameter and wall to lumen ratio.

Cryosectioning

Cryopreserved tissues were stored at -80°C until use. Sections of 8 µM thickness were made using a Cryocut tissue sectioner (Leica CM 1850). Cryosections were placed on poly-L-lysine coated microscope slides, air dried and stored at -80°C until further use.

Collagen Staining

A Masson's trichrome staining kit (Richard Allan Scientific; Kalamazoo, MI) was used to detect collagen deposition in the gastrocnemius muscle. Cryo-sections were stained following the manufacturer's instructions. Collagen deposition is displayed as a blue color. All images were captured with an Olympus FluoView 1000 light microscope (B&B Microscope Ltd.; Pittsburgh, PA). Measurement of collagen content was performed using Image J software (NIH free software download).

Reverse Transcription Polymerase Chain Reaction (PCR)

Total RNA was isolated from mouse gastrocnemius tissue using Trizol reagent (Invitrogen; Carlsbad, CA). Purified RNA (1 µg) was reverse transcribed using the Bio Rad iScript Reverse Transcription system (Hercules, CA) according to the manufacturer's instruction. To amplify specific gene sequences, primers were designed and obtained from Invitrogen (Carlsbad, CA). PCR amplification was performed for 35 amplification cycles in a Biorad DNA Engine Thermo-cycler (Hercules, CA). PCR products were resolved by gel electrophoresis in 1% agarose gels. PCR band images were recorded and analyzed using Imagelab software (Bio-Rad, Hercules, CA). The band intensities of the PCR product were normalized to GAPDH.

Real-Time PCR or qPCR

Reverse transcription of RNA occurs in the same manner as described for Reverse Transcriptase PCR. cDNA product, primers, nuclease free water and SYBR Green reaction mix (Qiagen; Gaithersburg, MD) were added to wells on a 96 well plate and the gene sequences were amplified for 55 cycles using a Roche Light Cycler.

Western Blotting

Gastrocnemius tissues were homogenized and lysed in radioimmunoprecipitation assay (RIPA) lysis buffer, supplemented with a protease inhibitor cocktail, phenylmethanesulfonylfluoride (PMSF) and sodium orthovanadate. Lysates were then sonicated for 5 seconds each and then centrifuged at 8,000 RPM for 10 minutes. Precipitates (protein) were then transferred to a new tube. Protein concentrations were determined via Bradford protein estimation assay to ensure equal protein loading, then samples were run on an SDS poly-acrylamide gel in Tris-glycine SDS buffer for proper protein separation and were then transferred electrophoretically onto a PVDF membrane overnight at 4 degrees. The membrane was blocked in a 5% milk TBST solution for 1 hour. All primary antibodies were diluted at a concentration of 1:1000 in TBST and membranes were incubated with primary antibody solution overnight at 4 degrees. After primary incubation, the membranes were washed three times with TBST solution then incubated with a secondary HRP conjugated antibody

solution for 1 hour at room temperature. Membranes were then washed three more times with TBST then developed using a chemiluminescent substrate in a BioRad Chemidoc (Hercules, CA). Band intensity was determined using BioRad ImageLab software. All proteins of interest were normalized to the values of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Statistical Analysis

Statistical analysis was performed using both Microsoft Excel and Primer of Biostatistics software. The Primer of Biostatistics software was used to perform a one way ANOVA followed by multiple comparisons analysis with Bonferroni correction. This analysis was performed on the laser Doppler perfusion, mean blood pressure and % collagen measurements to determine differences between the means of many groups. Microsoft Excel was used to perform a standard t-test to determine mean differences for all other assays. An alpha level of $p < 0.05$ was used to determine statistical significance. All values are presented as mean $\pm$ standard error of the mean.

Results

Plasma Homocysteine Levels

Using an anti-homocysteine antibody to detect total plasma homocysteine and homocysteinylated proteins on a dot blot, it was determined that control CBS+/- mice have a 42% greater plasma homocysteine concentration than control C57 mice and exercise intervention decreased Hcy levels down to control C57 levels. Exercised C57 mice also demonstrated a significant decline in plasma Hcy levels (Figure 6).

Body Mass

At baseline, CBS+/- have significantly reduced body weight when compared to C57 and all other mouse models (23.7 vs 27.3 g, respectively). CBS/MMP-9 double KO mice are normal bodyweight and not significantly different from any group except for CBS+/- mice. After exercise, CBS+/- mice gained a significant amount of body weight, but no change was observed in any other group. After exercise, CBS+/- mice were no longer significantly underweight compared to any other group except for post-exercise MMP-9. In CBS+/- mice, the weight of the tibialis anterior (TA) was significantly lower than what was observed in the C57 mice. Although exercise did not significantly increase the weight of the TA in the CBS+/- mice when compared to the pre-exercise weight, it was no longer significantly lower than the weight of C57 pre or post exercise TA muscles (Figure 7).

Blood Pressure Measurements

CBS+/- mice exhibit significantly higher mean blood pressures than C57 mice and all other groups (122.0 vs 83.8 mmHg respectively) at baseline. After 6 weeks, control CBS+/- did not see a significant change in blood pressure due to age, but it was still significantly higher than all other groups. However, with 6 weeks of exercise, mean blood pressure in CBS+/- mice significantly decreased when compared to pre-exercise conditions (122.0 vs 87.1 mmHg respectively), as well as CBS+/- control conditions and was no longer significantly different from any other group. CBS/MMP-9 mice are normotensive and did not display significant differences between any other groups except CBS+/- (Figure 8).

Hindlimb Perfusion

Perfusion of the femoral artery was significantly lower in CBS+/- control mice than every other control group. Perfusion of the femoral artery in CBS/MMP-9 mice was not different from any other group except CBS+/- . With exercise training, the perfusion was significantly increased from baseline in every group except for CBS/MMP-9, which showed no change. After exercise, perfusion in CBS+/- mice was not significantly different from any other post-exercise group, indicating that perfusion of the hind limb was corrected with exercise (Figure 9).

Wall to Lumen Ratio and Lumen Diameter

The wall to lumen ratio, which is inversely correlated to cardiovascular health, was significantly elevated in CBS+/- mice when compared to every other group at baseline. With exercise intervention, the wall to lumen ratio in CBS+/- mice was significantly reduced when compared to baseline (0.36 vs 0.45, respectively), indicating that exercise is causing beneficial remodeling of the vascular smooth muscle. However, it was still significantly higher than C57 mice at baseline and post-exercise (0.27, 0.24 respectively). No other group saw a significant change in wall to lumen ratio with exercise. CBS/MMP-9 mice were not significantly different from any other group at baseline, indicating that MMP-9 may be responsible for the observed remodeling in HHcy. The lumen diameter of the femoral artery was also significantly declined in CBS+/- mice when compared to C57 mice at baseline (0.204 mm vs 0.264 mm, respectively), but was significantly increased by exercise (0.242 mm). Post-exercise lumen diameter in CBS+/- mice was not significantly different from C57 pre or post exercise Figure 10).

Collagen Staining

To determine if the impairments in hindlimb perfusion translated to increased fibrosis of the hindlimb skeletal muscle, collagen staining was performed on mouse gastrocnemius sections. Indeed, control CBS+/- gastrocnemius tissues displayed significantly higher collagen content than

observed in every other group (14.0% collagen content vs 2.4% in C57 controls). After exercise intervention, CBS+/- collagen content significantly fell to 2.8%, making it insignificant from C57 controls or C57+exercise. FVB, MMP-9 and CBS/MMP-9 control groups were all slightly, but significantly higher than C57 controls. MMP-9 mice saw a decline in muscle collagen content with exercise training, but, besides CBS+/-, the other groups did not. Although CBS/MMP-9 control mice had significantly higher collagen content in their gastrocnemius than C57 at controls, they were not different from FVB and MMP-9 controls (Figure 11).

CBS +/- Mice Have Impaired Metabolic Activity in the Skeletal Muscle

Cytochrome C oxidase is the last enzyme in the electron transport chain and is crucial for oxidative metabolism. Using PCR, it was determined that control CBS+/- mice display 20% lower cytochrome C oxidase mRNA in the gastrocnemius muscle when compared to C57 controls, indicating lower skeletal muscle metabolic activity in CBS+/- mice. However, with exercise training, mRNA levels were significantly increased to the same levels as expressed in C57. There was no change observed in C57 with exercise (Figure 12).

Hyperhomocysteinemia Induces Myostatin

Western blotting analysis of the gastrocnemius tissue demonstrated that control CBS+/- mice have a 2 fold increase in myostatin levels when compared to

C57 controls, indicating that CBS+/- mice have impaired muscle growth compared to their wild type counterparts. Although myostatin demonstrated a general decline with exercise in CBS+/- mice, it was not quite significant ($p = 0.055$). Surprisingly, C57 mice demonstrated a significant increase in myostatin levels with exercise when compared to the control group. However, post-exercise myostatin levels in C57 and CBS+/- tissues were almost identical (1.35 vs 1.39 fold increase from C57 controls respectively), with no significant differences between the two values. CBS/MMP-9 control levels were not significantly different from C57 controls (Figure 13).

Exercise Increases Follistatin mRNA Expression

PCR analysis of the gastrocnemius tissues indicated that exercise significantly increased follistatin mRNA expression in C57 and CBS+/- muscle by 2.5 and 3 fold respectively, when compared to their non-exercise counterparts. However, although control CBS+/- mice displayed a trend for lower follistatin expression compared to C57 controls, the value was not significantly less ($p=0.08$). Values for CBS+/- mice with exercise were significantly higher than control C57 values, but not significantly different from C57 with exercise (Figure 13).

Hyperhomocysteinemia Induces Markers of Hypoxia

Western blotting revealed that control CBS+/- mice express significant increases in VEGF and iNOS protein when compared to C57 controls (1.32 and 1.44 fold increase, respectively). As expected, exercise increased both VEGF and iNOS in C57 mice compared to their control counterparts (1.20 and 1.38 fold increase, respectively). In exercised CBS+/- mice, VEGF was significantly reduced while iNOS did not change when compared to CBS+/- controls, with VEGF being reduced to C57 control levels. VEGF in CBS+/- exercise mice was also significantly lower than C57 exercise mice. CBS+/- exercise mice expressed greater iNOS than C57 controls as well, but it was not significantly different from exercised C57 (Figure 14).

Hyperhomocysteinemia Induces EMMPRIN and MMP-9

EMMPRIN protein, a known inducer of MMP's, was upregulated in CBS+/- control gastrocnemius muscle compared to C57 controls (1.53 fold increase). Exercise significantly reduced CBS+/--exercise EMMPRIN levels, while increasing C57 exercise levels compared to their non-exercise counterparts (1.30 and 1.37 fold increase over C57 controls, respectively). CBS+/- and C57 exercise groups were not significantly different from one another. Not surprisingly, MMP-9 mRNA expression was significantly higher in CBS+/- controls compared to C57 controls (0.171 vs 0.047 respectively) and was significantly reduced with exercise (0.030). However, there were no significant differences between the control and exercise

C57 groups or CBS+/- exercise. Interestingly, despite the difference in mRNA expression, MMP-9 protein expression followed a very similar pattern to EMMPRIN protein expression. MMP-9 was significantly higher (almost 2 fold) in CBS+/- controls than C57 controls and was significantly reduced with exercise. Exercise increased MMP-9 levels in the C57 exercise group and there was no significant difference between this group and the CBS+/- exercise group (Figure 15).

HHcy Promotes Inflammation in CBS+/- Skeletal Muscle

PPAR- γ protein expression, a pro-antioxidant factor, is significantly decreased in the skeletal muscle of CBS+/- controls compared to C57 controls (14% lower). Interestingly, it is significantly declined in CBS+/- and C57 exercise groups when compared to their respective control counterparts. Protein expression of IL-1 β , a pro-inflammatory cytokine, is significantly increased in CBS+/- control gastrocnemius tissue compared to C57 controls (46% higher). Interestingly, there is a trend suggesting that these molecules may increase in the muscle with exercise in both groups, but due to variability, neither of these values are significantly different from their controls (Figure 16).

Figure 6: Elevated plasma Hcy levels in CBS +/- mice are decreased with exercise. Representative dot blot image of Hcy levels in mouse plasma samples. Plasma Hcy levels were significantly higher in CBS NE compared to C57 NE ($p < 0.001$); these levels were decreased in CBS ET compared to CBS NE ($p < 0.001$). Plasma Hcy was also lowered in C57 ET mice compared with C57 NE ($p < 0.005$). Band density was quantitated using BioRad Image Lab software. Data are shown as $\pm$ SEM. $n = \geq 3$.

Figure 7: HHcy leads to decreased body mass and muscle mass, but is mitigated by exercise training. The above data show that A) CBS +/- mice have lower body weight compared to all other mouse groups used; B) exercise training mitigates the weight difference between CBS +/- and WT; and C) TA mass is significantly lower in CBS +/- NE when compared to C57 NE, but exercise training mitigates this weight difference. Data are displayed as mean $\pm$ SEM. $n \geq 3$; $p < 0.05$. * = different from corresponding control group; # = different from C57 NE.

Figure 8: Exercise training mitigates HHcy induced hypertension. The above data are a representation of mean blood pressure measures for all mouse groups at baseline, after 6 weeks of control and after 6 weeks of exercise. CBS +/- mice have significantly higher mean blood pressure than every other group at baseline and this is completely mitigated after 6 weeks of exercise. Data are represented as mean $\pm$ SEM. $n \geq 3$; $p < 0.05$. * = different from corresponding control group; # = different from C57 NE.

Figure 9: HHcy causes impaired hind limb perfusion that is mitigated with exercise training. Femoral artery perfusion is decreased in CBS NE compared to C57 NE and is significantly increased with exercise. Flux was measured using a laser Doppler imager. Data are represented as mean $\pm$ SEM. $n \geq 3$; $p < 0.05$; * = different from corresponding control group; # = different from C57 NE.

Figure 10: HHcy causes an increased wall to lumen ratio and a decreased lumen diameter, but these effects are reversed with exercise training. At baseline, CBS +/- mice have a significantly higher femoral artery A) wall to lumen ratio and significantly lower B) lumen diameter when compared to C57 pre-exercise. Exercise mitigates these effects. Data are represented as mean $\pm$ SEM. $n \geq 3$; $p < 0.05$.

Figure 11: Exercise training mitigates HHcy induced skeletal muscle fibrosis. Control CBS +/- mice have a significantly higher % of total collagen in the gastrocnemius than every other group. Exercise training reverses this. FVB, MMP-9 and CBS/MMP-9 control groups all have higher total collagen content than C57 NE. These are FVB based, which is a different mouse breed. Data are represented as mean $\pm$ SEM. $n \geq 3$; $p < 0.0005$ vs C57 NE and CBS ET.

Figure 12: HHcy causes decreased oxidative metabolism in skeletal muscle, but it is improved by exercise. Cytochrome C oxidase mRNA expression is lower in control CBS +/- gastrocnemius muscle compared to C57 control. Exercise training increases Cytochrome C oxidase expression in CBS +/- mice. The mRNA expressions were determined through densitometry analysis. Data are represented as mean $\pm$ SEM. $n \geq 4$; $p < 0.0005$; 0.05 , vs C57 NE and CBS ET, respectively. * = different from corresponding control group; # = different from C57 NE

Figure 13: HHcy alters skeletal muscle growth regulators and exercise training reverses the effects. A) Myostatin protein expression is elevated in CBS NE gastrocnemius compared to C57 NE and the expression is normalized with exercise. B) Follistatin expression is increased in C57 and CBS mice with exercise in comparison to their respective controls. Densitometry analysis was used for band quantitation. Data are represented as mean $\pm$ SEM. $n \geq 4$; $p < 0.01$, 0.05 for CBS NE vs C57 NE and CBS NE VS CBS ET, respectively for myostatin. $p < 0.01$ for CBS ET vs CBS NE for follistatin. * = different from corresponding control group; # = different from C57 NE

Figure 14: HHcy induces hypoxia in skeletal muscle. VEGF and iNOS protein expression are elevated in control CBS +/- mice when compared to C57 NE, indicating baseline hypoxia. Exercise decreases VEGF expression in CBS +/- mice to C57 NE levels. Band density was determined through densitometry analysis. Data are represented as mean $\pm$ SEM. $n \geq 4$; $p < 0.0001$, 0.0005 for CBS NE vs C57 NE and CBS NE vs CBS ET, respectively for VEGF. $p < 0.005$ for CBS NE vs C57 NE for iNOS.

Figure 15: Exercise training mitigates HHcy induction of EMMPRIN and MMP-9. A) EMMPRIN protein and MMP-9 B) protein and C) mRNA are induced in control CBS +/- gastrocnemius compared to C57 NE. Exercise causes a significant decrease in all three of these values. Data are represented as mean $\pm$ SEM. $n \geq 4$; $p < 0.005$, 0.05 for CBS NE vs C57 NE and CBS NE VS CBS ET, respectively for EMMPRIN. $p < 0.005$, 0.05 for CBS NE vs C57 NE and CBS NE VS CBS ET, respectively for MMP-9. $p < 0.05$, 0.01 for CBS NE vs C57 NE and CBS NE VS CBS ET, respectively for MMP-9 mRNA.

Figure 16: HHcy creates inflammatory environment in skeletal muscle.

A) PPAR- γ protein expression is reduced and B) IL-1 β protein expression is increased in CBS+/- mice compared with C57 NE. Data are represented as mean $\pm$ SEM. $n \geq 4$; $p < 0.0005$ for CBS NE vs C57 NE for PPAR- γ . $p < 0.005$ for CBS NE vs C57 NE IL-1 β .

Discussion

HHcy is implicated in diseases such as atherosclerosis, heart disease and diabetes and is thought to cause multi-organ damage (Bortolotto et al., 1999; Miller et al., 2000; Signorello et al., 2007; Sutton-Tyrrell et al., 1997; Zheng et al., 2013). Recent research has demonstrated that HHcy results in impaired responsiveness to ischemia/reperfusion injury, lower muscle strength and functionality, increased fatigability and lower muscle ATP levels (Swart et al., 2013; Sudhakar Veeranki et al., 2014; S. Veeranki et al., 2015). However, the effects of regular exercise on cardiovascular disease and skeletal muscle function are known to be very beneficial. It has also been established that exercise has the capability of reducing homocysteine levels (Neuman et al., 2013; Randeve et al., 2002). Nevertheless, despite the abundance of research on HHcy, there is very little information available regarding the molecular changes that HHcy induces in the skeletal muscle tissue and there is even less information available regarding the effects of exercise on these changes. The present study demonstrates that HHcy induces skeletal muscle fibrosis through induction of inflammatory factors and impairment of perfusion. This study demonstrates that exercise is capable of attenuating most of these effects.

In this study, CBS +/- (HHcy) mice displayed a 13.2% decrease in body weight, decreased skeletal muscle weight and a 42% increase in plasma homocysteine levels when compared to C57 (WT) mice. These findings agree with previous clinical studies that found patients with HHcy due to genetic defects to have a significant reduction in body weight and muscle mass (Kalra et al.,

1985; Kanwar et al., 1976). Dot blot analysis determined that CBS +/- mice do in fact have a 42% greater concentration of plasma homocysteine and homocysteinylated proteins when compared to C57 mice. CBS +/- mice also demonstrated a significant elevation in mean blood pressure, placing them well above normal levels (122 mmHg). Not surprisingly, this elevation in blood pressure was accompanied by significant reductions in luminal diameter and perfusion of the femoral artery as well as a significant increase in wall to lumen ratio. This data indicates that HHcy is likely inducing adverse remodeling of the vascular smooth muscle, causing a thicker and less compliant vascular wall which will cause elevated blood pressure. Our data agrees with previous findings from our laboratory that suggests that CBS +/- mice demonstrate a blunted response to ischemia/reperfusion injury after femoral artery ligation (Veeranki et al., 2014). The CBS +/- mice demonstrated impaired development of collateral vessels in the ischemic hind limb due to impaired angiogenesis, which was caused by blunted elevation of pro-angiogenic factors such as VEGF, HIF1- α and PGC-1 α . Our findings are also supported by other studies which demonstrate that HHcy is associated with increased intraluminal thrombus thickness, aortic stiffness, systolic hypertension and myocardial impairment (Bortolotto et al., 1999; Sutton-Tyrrell et al., 1997; Wiernicki et al., 2011; Zheng et al., 2013). Bortolotto et al. demonstrated that in 236 hypertensive patients, individuals with 2 or 3 sites of clinical vascular disease had significantly higher plasma homocysteine levels than hypertensive patients with no clinical vascular disease (Bortolotto et al., 1999). Accompanying impaired angiogenesis and vascular

disease, other studies demonstrate that Hcy promotes the formation of ROS and diminishes nitric oxide production in the vasculature, which are both associated with impaired endothelial responsiveness and hypertension (Signorello et al., 2007; Tyagi et al., 2009).

With HHcy causing impaired perfusion to the hind limb and skeletal muscle weakness, it would seem likely that the skeletal muscle in CBS +/- mice would display signs of fibrosis. Indeed, this study demonstrated that the CBS +/- gastrocnemius muscle displayed a 6 fold increase in skeletal muscle collagen deposition when compared to C57 controls. Excessive collagen deposition, which is a hallmark of muscular fibrosis, leads to increased rigidity and decreased functionality of skeletal muscle (Dodd et al., 2014; Gillies & Lieber, 2011). These findings support other studies that suggest that HHcy induces skeletal muscle weakness and impaired functionality, which could clearly be caused by muscular fibrosis (Swart et al., 2013; Veeranki et al., 2015).

Interestingly, this data demonstrates that with implementation of a 6 week treadmill exercise program these CBS +/- mice demonstrate increased weight gain, muscle mass, femoral artery perfusion and lumen diameter. This study also found a complete reversal of the plasma homocysteine levels, hypertension and collagen deposition as well as a decrease in the CBS +/- wall to lumen ratio. Furthermore, CBS/MMP-9 double KO mice did not exhibit any of these issues. CBS/MMP-9 mice are normal weight and have normal blood pressure, perfusion and wall to lumen ratio as well as significantly lower collagen deposition in the skeletal muscle than observed in the CBS +/- mice. Although there were

insufficient plasma samples for statistical analysis, it appears that the CBS/MMP-9 mice have similar homocysteine levels to the CBS +/- mice (1.45 vs 1.43 fold increase compared to C57 control, respectively). This data suggests that Hcy elicits the majority of its associated pathologies through hyperactivity of MMP-9, which is known for its ability to degrade the ECM.

The findings that exercise reverses the effects of HHcy on skeletal muscle dysfunction agree with other studies that exercise decreases or prevents increases in plasma Hcy levels (Neuman et al., 2013; Randeva et al., 2002). Data from this study supports findings from the lab's previous study, which suggests that swimming exercise can attenuate the effects of HHcy on the down-regulation of mitochondrial transcription factors and ATP production in the skeletal muscle (Veeranki et al., 2015). This evidence suggests that CBS +/- mice have impaired skeletal muscle metabolism when compared to control mice and that exercise can improve its metabolic function. This study further supports this theory by demonstrating that CBS +/- mice have a decrease in gastrocnemius cytochrome c oxidase mRNA expression compared to C57 controls. It was found that exercise completely reverses this metabolic deficit.

With a decreased mass, elevated fibrosis and impaired metabolic activity in CBS +/- skeletal muscle, I wanted to determine whether or not the skeletal muscle growth was being impaired by changes in molecular growth factors. The data suggests that CBS +/- gastrocnemius muscle had a 2 fold increase in expression of myostatin protein, a muscle growth inhibitor, when compared to C57 controls. CBS/MMP-9 myostatin expression was significantly lower than

levels in CBS +/- mice and was not different from levels in C57 controls, once again confirming that MMP-9 induction is likely the mechanism of action for HHcy. As expected, myostatin levels in CBS +/- mice were significantly reduced with exercise and were reduced to the same levels as seen in exercised C57 mice, which correspond well with the findings that exercise increases follistatin mRNA expression in CBS +/- mice. Interestingly, there was an observed increase in myostatin levels in C57 exercise mice compared to their control counterparts. The observed significant increase in follistatin mRNA expression, a known inhibitor of myostatin, in C57 mice with exercise suggests that myostatin may be over-expressed in C57 mice post-exercise due to positive feedback loops from myostatin inhibition during exercise. This exercise protocol was designed for moderate to high intensity by the end of the protocol to ensure muscular hypertrophy as seen in some resistance training programs. Findings that myostatin increased in the skeletal muscle of C57 mice with exercise training is supported by findings from Willoughby, 2004. This author found that 12 weeks of resistance training in 22 healthy males resulted in elevated body mass, muscle mass and strength, but also demonstrated increases in myostatin mRNA, myostatin and follistatin-like related gene, another inhibitor of myostatin (Willoughby, 2004). Furthermore, unchecked myostatin levels, as seen in control CBS +/- mice, has been shown to directly increase fibroblast activity, which in turn, increases macrophage activity (McCroskery et al., 2005; Vidal et al., 2008). Seeing as both macrophages and fibroblasts are implicated in muscular fibrosis,

it makes sense that control CBS +/- mice, which over-express myostatin, would have fibrotic muscle tissue.

The findings that VEGF and iNOS are induced in the gastrocnemius muscle of control CBS+/- mice and exercised C57 mice is not surprising, as both are commonly induced during states of hypoxia, including exercise (Forsythe et al., 1996; Hambrecht et al., 1999; Richardson et al., 1999; Richardson et al., 2000; Shweiki, Itin, Soffer, & Keshet, 1992; Vogt et al., 2001). However, it was interesting to see that VEGF was decreased to C57 control levels in exercise CBS+/- mice. A study from Richardson et al. demonstrates that exercise training causes a reduction in VEGF stimulation when exposed to hypoxic conditions, suggesting that VEGF stimulation has a negative feedback mechanism (Richardson et al., 2000). It is plausible that CBS +/- muscle tissue has become insensitive to VEGF stimulation and with training, angiogenesis has occurred through an elevated stimulation of other angiogenic factors such as fibroblast growth factor, causing a decline in VEGF necessity. Furthermore, an increased capillary to fiber ratio, as observed with exercise training, would decrease the basal level of hypoxia in the muscle tissue, resulting in a decrease in VEGF production.

As expected with HHcy, MMP-9 mRNA and protein expression as well as protein expression of its inducer, EMMPRIN, were all significantly elevated in CBS+/- mice when compared to C57 controls. Interestingly, EMMPRIN and MMP-9 protein levels are both significantly increased with exercise training in C57 mice. Although MMP-9 has been associated with numerous diseases, it has

also been demonstrated that MMP-9 assists with satellite cell translocation (Kherif et al., 1999; Lewis et al., 2000; Zimowska et al., 2008) and may be required for the onset of angiogenesis (Bellafiore et al., 2013; Mackey et al., 2004; Rullman et al., 2009). However, this does not explain why EMMPRIN and MMP-9 protein expression is decrease in exercise CBS+/- mice compared to control CBS+/- . Seeing as exercise decreased these values to levels that are almost identical to exercised C57 values, it would seem that basal levels of these proteins in CBS+/- mice were more than sufficient to accomplish necessary growth associated tasks for exercise, allowing for normalization of their values. Although protein expression of MMP-9 went up with exercise in C57 mice, the data demonstrates a decrease in MMP-9 mRNA values. Since the animals were sacrificed 2 days post-exercise to allow time for blood pressure and ultrasonography data to be collected, it is plausible that the elevated MMP-9 protein expression elicited a reduction in MMP-9 mRNA via negative feedback during that time frame.

To assess other markers of inflammation, changes in IL-1 β , a pro-inflammatory cytokine, and PPAR- γ , a pro-antioxidant, protein expressions in the gastrocnemius tissue were measured. In CBS+/- mice, IL-1 β was elevated and PPAR- γ was decreased when compared to C57 control mice, as expected with HHcy. It is well known that Hcy induces inflammation and it has been demonstrated that it also inhibits PPAR- γ (Mujumdar et al., 2002). Interestingly, this data suggests that PPAR- γ is decreased with exercise in both C57 and CBS +/- groups. These results agree with a data from Tunstall et al. that demonstrated

a significant reduction in PPAR- γ in human vastus lateralis muscle after 9 days of 60 minute cycling bouts (Tunstall et al., 2002). With exercise requiring inflammation for proper growth and repair during the recovery phase, as seen in elevated levels of MMP-9 and EMMPRIN, it is plausible that a potent pro-antioxidant like PPAR- γ would be down-regulated to allow for adequate induction of the inflammatory process. Although the elevations are insignificant, the slight increases in IL-1 β observed with exercise would support this theory.

In summary, results of this study indicate that HHcy causes muscle dysfunction that is induced through multiple anti-proliferative and inflammatory factors such as myostatin, EMMPRIN and MMP-9. It would appear that MMP-9 is a primary pathway through which HHcy causes pathological conditions since CBS/MMP-9 double KO mice have elevated Hcy levels, but do not display any of the pathological conditions that are witnessed in CBS+/- mice. Furthermore, exercise was capable of mitigating most of the pathological complications associated with HHcy. Clearly, further research on the exact mechanism of action for HHcy induced skeletal muscle dysfunction needs to be conducted.

CHAPTER V

THE EFFECT OF HYPERHOMOCYSTEINEMIA ON MACROPHAGE DIFFERENTIATION AND SKELETAL MUSCLE INFILTRATION

Introduction

Homocysteine has been widely identified as a risk factor in numerous disease states. HHcy, demonstrating plasma Hcy levels $>15 \mu\text{mol/L}$, has known prevalence in hypertension, stroke, cardiovascular disease and skeletal muscle dysfunction (Bortolotto et al., 1999; Hayden & Tyagi, 2004; Mujumdar et al., 2002; Signorello et al., 2007; Sutton-Tyrrell et al., 1997; Tyagi et al., 2005; Veeranki & Tyagi, 2013). Research has shown a direct correlation between plasma Hcy levels and aortic pulse wave velocity, intraluminal thrombus thickness and arterial stiffness.

It is well established that Hcy elicits many of its detrimental effects through increased ROS production and through induction of MMP-9 (Hayden & Tyagi, 2004; Lee et al., 2012; Signorello et al., 2007; Tyagi et al., 2009). MMP-9 is an endoproteinase that is known to degrade components of the extracellular matrix, such as elastin and collagen type IV. Excessive MMP-9 activity has been associated with endothelial dysfunction and vascular

remodeling, leading to diseases such as atherosclerosis (Onal et al., 2009; Wiernicki et al., 2011; Yasmin et al., 2005). More recently it has been discovered that MMP-9 production and activity are increased through an EMMPRIN mediated pathway involving NF- κ B (Ge et al., 2007; Major et al., 2002; Reddy et al., 2010; Yoon et al., 2005; Zhu et al., 2005). EMMPRIN is a glycoprotein that can be induced through inflammatory factors such as IL-18. In a study involving human carotid endarterectomy specimens, significant EMMPRIN immunoreactivity was displayed in atherosclerotic carotid lesions, arterial plaque and in macrophage infiltrates of the atherosclerotic intima (Yoon et al., 2005). This same study also examined the effects of atherogenic pro-inflammatory cytokines (TNF- α , IL-6 and IL-1 β) on cultured human bone marrow monocytes and discovered increased expression of EMMPRIN protein and increased MMP-9 activity.

With the numerous pathological effects of HHcy revolving around ECM degradation, vascular remodeling, cardiovascular disease and inflammation, it is important to ponder the effects of Hcy on macrophages, the body's main innate immune cells. It is well established that macrophages have two specific subtypes, denoted as M1 and M2. The M1 phenotype is the destructive, "classically activated" subset, while the M2 is called the "alternatively activated" subset, associated with growth and repair (Mills, 2012; Sica & Mantovani, 2012). Although classically activated macrophages are important for fighting infections, previous research has demonstrated that excessive macrophage differentiation into the M1 phenotype can cause chronic adipocyte inflammation, neurotoxicity

to injured nerves and damage the arterial vasculature (Kigerl et al., 2009; Oh et al., 2012; Tavakoli & Asmis, 2012; Zeyda et al., 2007). It has been demonstrated that M1 macrophages elicit their inflammatory and damaging effects through a rapid production of ROS, NO and MMP-9 (Mills, 2012; Sica & Mantovani, 2012; Woo, Lim, & Kim, 2004). Interestingly, It has been demonstrated that Hcy induces vascular endothelial growth factor expression and monocyte chemoattractant protein-1 via an NF- κ B dependent pathway in THP-1 macrophages, which are both associated with vascular disease progression (Wang et al., 2001). There are several markers that have been used to detect M1 and M2 macrophages, however, Vogel et al. clearly demonstrate that CD40, a cell surface co-stimulatory protein found on macrophages, is a highly effective marker for detection of the M1 phenotype (Vogel et al., 2013).

In the skeletal muscle, macrophages are crucial for proper growth and repair. Once macrophages phagocytose muscle cell debris, they shift towards an anti-inflammatory profile, causing the release of TGF- β and bFGF, which stimulates muscle satellite cell migration (Arnold et al., 2007; Robertson et al., 1993). Injury to the skeletal muscle elicits significant increases in macrophage infiltration for 3 days post injury then these levels begin to subside by day 6 (Pimorady-Esfahani et al., 1997). If monocyte recruitment to the site of damaged skeletal muscle is interrupted within the first 24 hours post-injury, the repair process is completely attenuated. Inflammatory macrophages stimulate myogenic cell proliferation, while anti-inflammatory macrophages stimulate myogenic cell differentiation into myotubes (Arnold et al., 2007). Complete

removal of macrophages after muscular injury decreases the diameter of regenerating muscle fibers (Arnold et al., 2007; Chazaud et al., 2009). Research demonstrates that exercise stimulates the phagocytic activity of macrophages (De la Fuente et al., 1990; Ferrandez & De la Fuente, 1999). However, the effects of exercise on macrophage differentiation and infiltration into the skeletal muscle, especially in a chronically inflammatory environment, have not been well-documented. Kawanishi et al. demonstrated that mice being fed a high fat diet display a greater ratio of adipose tissue M1/M2 macrophages compared to normal diet controls (Kawanishi et al., 2010). After 16 weeks of exercise training, there was a decreased ratio of M1/M2 macrophages in the high fat diet group due to increased presence of M2 macrophages. This was accompanied by a decrease in TNF- α mRNA expression, which is only expressed by M1 macrophages.

There have been no other studies that determine whether Hcy alters macrophage differentiation through EMMPRIN or if chronic HHcy leads to increased M1 macrophage infiltration into skeletal muscle. Therefore, it is the purpose of this study to determine the effects of homocysteine on the phenotypic differentiation of macrophages and whether or not it increases M1 macrophage presence in skeletal muscle tissue.

Methods

Chemicals and Antibodies

L-homocysteine was purchased from Sigma-Aldrich (St. Louis, MO). EMMPRIN, CD40, F4/80 and all HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX). GAPDH antibody was purchased from EMD Millipore (Billerica, MA). MMP-9 and TNF- α antibodies were purchased from Abcam (Cambridge, MA) and fluorescent secondary antibodies were purchased from Life Technologies (Carlsbad, CA).

Animal Care and Exercise Protocol

C57 and CBS +/- mice were used for this study. Animal care and the exercise protocol are as previously described in Chapter IV.

Cell Culture

J774A.1 and Raw 264.7 murine macrophages cell lines were obtained from ATCC (Manassas, VA). Cells were grown in 75cm² flasks using ATCC 30-2002 Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% final volume penicillin-streptomycin solution. Cells were maintained in a 37 °C incubator with 5% CO₂ and passaged by scraping cells into fresh medium per ATCC recommendations.

Cell Treatment

Cells were plated in 6 well plates at a count of 750K cells per well for harvesting of protein or in 8 well chamber slides at a count of 150K cells per chamber for Immunocytochemistry. After 24 hours of incubation, to allow for proper plate adherence, the medium was aspirated and fresh medium was added to each well. Homocysteine treatment was added directly to the medium at a final concentration of 500 μ M for Raw 264.7 cells and 100 μ M for J774A.1 cells. Cells were then incubated with Hcy treatment for 24 hours.

Western Blot Analysis

Gastrocnemius tissue was used for protein analysis. For cell culture experiments, cells were harvested 24 hours after Hcy treatment for protein analysis. The DMEM medium was aspirated and cells were then washed twice with PBS. Cells or gastrocnemius tissues were then lysed in RIPA lysis buffer, supplemented with a protease inhibitor cocktail, PMSF and sodium orthovanadate, for 15 minutes on ice. For cell culture, each well was then scraped and contents were transferred to an Eppendorf tube. Cell culture and gastrocnemius lysates were then sonicated for 5 seconds each and then centrifuged at 8,000 RPM for 10 minutes. Precipitates were then transferred to a new tube. Protein concentrations were determined via Bradford protein estimation assay to ensure equal protein loading then samples were run on an SDS poly-acrylamide gel in Tris-glycine SDS buffer for proper protein separation

and were then transferred electrophoretically onto a PVDF membrane overnight at 4 degrees. The membrane was blocked in a 5% milk TBST solution for 1 hour. All primary antibodies were diluted at a concentration of 1:1000 in TBST and membranes were incubated with primary antibody solution overnight at 4 degrees. After primary incubation, the membranes were washed three times with TBST solution then incubated with a secondary HRP conjugated antibody solution for 1 hour at room temperature. Membranes were then washed three more times with TBST then developed using a chemiluminescent substrate in a BioRad Chemidoc (Hercules, CA). Band intensity was determined using densitometry analysis.

DCFDA ROS Assay

To detect changes in ROS production levels between control and Hcy treated cells, cells were aspirated 24 hours post treatment and fresh medium containing 20 μ M 2',7' –dichlorofluorescein diacetate (DCFDA) was added to each well. For EMMPRIN inhibition ROS assay, anti-EMMPRIN antibody was diluted 1:200 in complete DMEM medium and added 2 hours prior to treatment. Cells were incubated with DCFDA at 37 degrees for 30 minutes. The medium was then aspirated and each well was washed 3 times with PBS. Fresh PBS was then added to each well and the cells were imaged using confocal microscopy.

Immunocytochemistry

After cell treatment, the medium was aspirated from each well of the chamber slides and they were then washed twice with PBS. Cells were then fixed using a 4% paraformaldehyde solution for 15 minutes at room temperature, which was followed by 3 washes with PBS. A PBS solution with 3% BSA and 0.3% Triton X-100 was used to block non-specific binding and permeabilize the cells for 1 hour. Primary antibody was then added in a concentration of 1:50 for CD40 and 1:200 for MMP-9 in a PBS solution containing 1% BSA and 0.1% TX-100 and was incubated overnight at 4°C. The chamber slides were aspirated, washed 3 times and then incubated with fluorescent secondary antibody (1:200) for 2 hours at room temperature. Chambers were then washed 3 times and then a DAPI staining solution was added for 5 minutes, followed by 3 more washes. Confocal Microscopy was then used to detect fluorescence.

EMMPRIN Antibody Treatment

To assess the role of EMMPRIN on M1 macrophage differentiation, prior to homocysteine treatment, EMMPRIN antibody was added in a final concentration of 1:200 directly to the cell culture medium to inhibit EMMPRIN function. Cells were allowed to incubate with antibody for 2 hours and then the homocysteine treatment was administered as described above.

Immunohistochemistry

Tissues were cryo-sectioned onto poly-L-lysine coated glass microscope slides and allowed to air dry. Slides were stored at -80°C until use. Slides were dipped in deionized water to remove tissue freezing medium from slide. Tissue sections were then fixed using a 4% paraformaldehyde solution for 15 minutes at room temperature, which was followed by 3 washes with PBS. A PBS solution with 3% BSA and 0.3% Triton X-100 was used to block non-specific binding and permeabilize the cells for 1 hour. Primary antibody was then added in a concentration of 1:50 in a PBS solution containing 1% BSA and 0.1% TX-100 and was incubated overnight at 4°C. The slides were then washed 3 times and then incubated with fluorescent secondary antibody (1:200) for 2 hours at room temperature. Slides were then washed 3 times and then a DAPI staining solution was added for 5 minutes, followed by 3 more washes. Confocal Microscopy was then used to detect fluorescence. Fluorescent intensity was then quantitated using Image J software (NIH).

RESULTS

Hcy treatment induces EMMPRIN expression

Since EMMPRIN is suggested to be an inducer of matrix metalloproteinases, we decided to look at changes in EMMPRIN protein expression with Hcy treatment in these cells lines. Western blotting was used to determine changes in EMMPRIN expression. EMMPRIN was significantly increased in RAW 264.7 cells with 24 hours of 500 μ M Hcy treatment when compared with controls ($p < .001$). In J774A.1 cells, a significant induction ($p < .05$) of EMMPRIN protein with 24 hours of 100 μ M Hcy treatment was also observed (Figure 17); $n=3$.

Hcy treatment induces ROS production in macrophages

Since M1 macrophages are associated with increased reactive oxygen species (ROS) production, we utilized a DCFDA (2',7' -dichlorofluorescein diacetate) assay to determine if Hcy treatment causes increased ROS production in both strains of murine macrophages. Both RAW 264.7 and J774A.1 cells demonstrated increased ROS production ($p < .05$; $p < .01$) after 24 hours of 500 μ M and 100 μ M Hcy treatment respectively, when compared with controls (Figure 18); $n=3$.

Hcy treatment induces MMP-9 protein

To determine whether or not MMP-9 was elevated in Hcy treated cells, immunocytochemistry (ICC) was performed on both cell lines to determine changes in expression of MMP-9 protein. After 24 hours of 100 μ M Hcy treatment, the J774A.1 cells displayed a significantly higher ($p < .05$) production of MMP-9 protein when compared with controls. Raw 264.7 cells incubated with 500 μ M Hcy treatment for 24 hours also displayed elevated levels of MMP-9 protein ($p < .05$). Fluorescent intensity was measured to quantitate these increases in MMP-9 production (Figure 19); $n=3$.

HCY Treatment Induces CD40 in murine macrophages

To determine the effect of Hcy on macrophage phenotype differentiation, J774A.1 and Raw 264.7 cells (mouse macrophage cell lines) were stimulated with Hcy treatment for 24 hours. Cells were harvested and lysed for protein analysis and analyzed through western blotting. CD40 was significantly increased ($p < 0.05$) in J774A.1 cells with 100 μ M Hcy treatment. The Raw 264.7 cells were not as sensitive to Hcy treatment as the J774A.1 cell line, but displayed a significant 2.24 fold increase ($p < 0.0001$) in CD40 protein with 500 μ M Hcy treatment (Figure 20); $n=3$.

Inhibiting EMMPRIN activity decreases the M1 macrophage phenotype

In order to determine if EMMPRIN is involved in differentiation of macrophages into the M1 phenotype, we decided to see if blocking EMMPRIN function would impact CD40 protein expression. RAW 264.7 and J774A.1 cells were incubated with anti-EMMPRIN antibody for 2 hours prior to no HCY treatment or 24 hours of 500 μ M and 100 μ M HCY treatment respectively. Raw 264.7 cells receiving anti-EMMPRIN antibody pretreatment with no Hcy treatment had significantly reduced ($p < .001$) CD40 protein expression compared to control. Cells receiving pre-treatment with anti-EMMPRIN antibody and treatment with Hcy also displayed a decreased expression of CD40 ($p < .005$). No difference was found between the two treatment groups. J774A.1 cells receiving anti-EMMPRIN antibody pretreatment with no Hcy treatment had significantly lower ($p < .005$) CD40 protein expression than controls. Cells receiving pre-treatment with anti-EMMPRIN antibody and treatment with Hcy also displayed a decreased expression of CD40 ($p < .005$). No difference was found between the two treatment groups (Figure 21); $n=3$.

EMMPRIN Inhibition Reduces ROS Production in J774A.1 Cells

Pre-treatment of J774A.1 cells led to a significant decline in ROS production when compared to controls. As expected, 100 μ M Hcy treatment elicited a significant, almost 2 fold, increase in ROS production compared to control. However, when Hcy treated cells were pre-treated with anti-EMMPRIN

antibody, ROS production was reduced to control levels. This indicates that EMMPRIN mediates Hcy induced ROS production in J774A.1 macrophages (Figure 22); n=3.

Exercise Decreases M1 Macrophage Infiltration in CBS+/- Skeletal Muscle

F4/80 is a cell surface protein that is only found on macrophages and monocytes. It is commonly used to detect macrophage populations within other tissues. TNF- α is present in skeletal muscle, but it has been documented that the vast majority of TNF- α expressed in skeletal muscle is produced by macrophages (Hamada, Vannier, Sacke, Witsell, & Roubenoff, 2005). Co-localization of F4/80 and TNF- α is indicative of an M1 macrophage population since TNF- α is only expressed by M1 macrophages. Immunohistochemistry for co-localization of TNF- α and F4/80 protein expression in gastrocnemius muscle sections indicated that TNF- α and F4/80 protein expression were significantly increased in CBS +/- mice when compared to C57 controls and exercise intervention caused a significant decline in CBS +/- TNF- α expression. Although F4/80 expression did not significantly decrease in CBS +/- mice with exercise, it was no longer significantly different from C57 control values, indicating a slight reduction in the number of macrophages. Although TNF- α demonstrated a slight decline in C57 mice with exercise, the values were not significant when compared with C57 control. No changes in F4/80 protein expression were observed in C57 mice with exercise (Figure 24); n=4.

Figure 17: Hcy increases EMMPRIN expression in macrophages. EMMPRIN protein expression is significantly increased in A) Raw 264.7 cells and B) J774A.1 cells by 24 hours of 500μM or 100μM Hcy treatment, respectively. Bands were quantitated using densitometry. Data is presented as the means $\pm$ SEM. n = 3; p < 0.001; p < 0.05 respectively.

Figure 18: Hcy increases ROS production in macrophages. ROS production is significantly increased in A) Raw 264.7 cells and B) J774A.1 cells by 24 hours of 500μM or 100μM Hcy treatment, respectively. DCFDA assay was used to detect ROS production. Data is presented as the means $\pm$ SEM. $n = 3$; $p < 0.05$; $p < 0.01$, respectively.

Figure 19: Hcy increases MMP-9 expression in macrophages. MMP-9 expression is significantly increased in A) Raw 264.7 cells and B) J774A.1 cells by 24 hours of 500µM or 100µM Hcy treatment, respectively. Immunohistochemistry was used to detect MMP-9. Data is presented as the means $\pm$ SEM. n = 3; p < 0.05; p < 0.05, respectively.

(Winchester et al., 2015)

Figure 20: Hcy causes macrophages to shift towards an M1 phenotype.

CD40 protein expression, a marker for M1 macrophages, is significantly increased in A) Raw 264.7 cells and B) J774A.1 cells by 24 hours of 500μM or 100μM Hcy treatment, respectively. Quantification was performed through densitometry. Data is presented as the means $\pm$ SEM. $n = 3$; $p < 0.0001$; $p < 0.05$, respectively.

Figure 21: Inhibition of EMMPRIN prevents M1 macrophage shift.

CD40 protein expression, is significantly increased in A) Raw 264.7 cells and B) J774A.1 cells by inhibition of EMMPRIN with or without 24 hours of 500μM or 100μM Hcy treatment, respectively. Quantification was performed through densitometry. Data is presented as the means $\pm$ SEM. $n = 3$; $p < 0.001$; $p < 0.005$, respectively for Ab and Hcy 500μM + AB groups vs control in Raw 264.7 cells. $p < 0.005$; $p < 0.005$, respectively for Ab and Hcy 100μM + AB groups vs control in J774A.1 cells.

Figure 22: Inhibition of EMMPRIN decreases ROS production in macrophages. ROS production is significantly decreased in J774A.1 cells by inhibition of EMMPRIN with or without 24 hours of 100μM Hcy treatment. Quantification was performed through densitometry. Data is presented as the means $\pm$ SEM. $n = 3$; $p < 0.001$, 0.05 and 0.0005 for Hcy 100μM vs Control, Ab vs Control and Hcy 100μM + Ab vs Hcy 100μM, respectively.

(Winchester et al., 2015)

Figure 23: Schematic representation of M1 shift through Hcy treatment.

Figure 24: Exercise decreases the number of M1 macrophages in CBS +/- skeletal muscle. TNF-α (M1) and F4/80 (macrophage marker) expression are increased in CBS +/- skeletal muscle. Exercise decreases TNF-α expression in CBS +/- mice, indicating a lower population of M1. Data is presented as the means $\pm$ SEM. $n = 4$; $p < 0.05$ for CBS NE vs C57 NE for F4/80. $p < 0.01$, 0.01 for CBS NE vs C57 NE and CBS ET vs CBS NE, respectively, for TNF-α.

DISCUSSION

The primary finding of this present study is that Hcy treatment causes macrophages to differentiate into a “classically activated,” M1 phenotype as determined by increased CD40 and MMP-9 expression, as well as elevated ROS production. It was found that inhibiting EMMPRIN activity, an inducer of MMP-9, resulted in reduced levels of CD40 in murine macrophages. This finding suggests that Hcy induced macrophage differentiation into M1 phenotype is facilitated, at least in part, by an EMMPRIN mediated pathway.

Due to its strong association with a variety of pathological conditions, many of the consequential effects of HHcy are well-explored (Bortolotto et al., 1999; Naess et al., 2013; Signorello et al., 2007; Tyagi et al., 2005; Veeranki & Tyagi, 2013; Zheng et al., 2013). Hcy associated vascular inflammation and remodeling is largely due to increased ROS (Hayden & Tyagi, 2004; Signorello et al., 2007) and MMP-9 production (Moshal et al., 2006; Tyagi et al., 2009) as well as an inhibition of peroxisome proliferator-activated receptor- γ (PPAR- γ), a pro-antioxidant (Mujumdar et al., 2002). MMP-9 is activated by Hcy through an extracellular signal-regulated kinase (ERK) pathway in vascular endothelial cells (Moshal et al., 2006; Tyagi et al., 2009) and is thought to be a primary factor in vascular remodeling and arterial stiffness (Onal et al., 2009; Wiernicki et al., 2011; Yasmin et al., 2005). However, studies have shown that extracellular matrix metalloproteinase inducer (EMMPRIN) activation not only correlates with MMP activity, but may be largely responsible for increased synthesis of MMP-9 (Ge et al., 2007; Major et al., 2002; Reddy et al., 2010; Yoon et al., 2005; Zhu et

al., 2005). It was found that co-localization of macrophages with EMMPRIN and MMP-9 occurs in areas of vascular inflammation. Wang et al. suggest that Hcy elicits monocyte chemotaxis through induction of monocyte chemoattractant protein-1 (Wang et al., 2001). Moreover, it was found that a high concentration of Hcy enhances MMP-9 production in murine macrophages (Lee et al., 2012). These findings led to further investigations to determine whether a pathological concentration of Hcy leads to macrophage differentiation into the classically activated, M1 phenotype.

CD40, a co-stimulatory protein, is a commonly used marker of M1 macrophages that has been described as a prevalent indicator of the M1 phenotype (Aron-Wisnewsky et al., 2009; Munder et al., 1998; Vogel et al., 2013). The findings that a pathological dose of Hcy induces CD40 and MMP-9 protein expression in both J774A.1 and Raw 264.7 murine macrophage cell lines suggest that Hcy is causing a shift towards the M1 phenotype. Our data corresponds with another study which demonstrates that Hcy enhances MMP-9 production in J774A.1 cells via ERK and Akt signaling pathways (Lee et al., 2012). Interestingly, other studies demonstrate that the pro-inflammatory cytokine, IL-18, induces MMP-9 in cardiomyocytes via EMMPRIN activation of a PI3K dependent Akt pathway (Reddy et al., 2010; Weisser et al., 2013). The present study demonstrates that 0.1 or 0.5 mM Hcy treatment significantly induces EMMPRIN protein synthesis in both J774A.1 and Raw 264.7 macrophages respectively. It has been established that EMMPRIN induction is strongly associated with inflammatory conditions. EMMPRIN is readily expressed

on human carcinoma cell surfaces, atheromas, pro-inflammatory cytokine stimulated bone marrow monocytes and in atherosclerotic carotid lesions (Gabison, Hoang-Xuan, Mauviel, & Menashi, 2005; Guo et al., 2000; Major et al., 2002; Yoon et al., 2005). Due to its close association with these inflammatory pathologies, I believe that EMMPRIN is at least partially involved in macrophage differentiation into the M1 phenotype. To verify this, the effects of EMMPRIN inhibition on CD40 expression were examined. As hypothesized, the inhibition of EMMPRIN led to a decreased induction of CD40 in both murine macrophage cell lines, with and without Hcy treatment. This suggests that M1 differentiation by Hcy is at least partially reliant upon an EMMPRIN mediated pathway.

Homocysteine is associated with increased ROS production in various cell types due to its auto-oxidative properties and through the production of mixed disulfides (Hayden & Tyagi, 2004; Signorello et al., 2007). Interestingly, elevated oxidative stress levels may result in a destructive M1 macrophage phenotype, as detected by increased release of iNOS, TNF- α and pro-inflammatory cytokines. Furthermore, it was found that increased EMMPRIN expression, resulting in activation of MMP-9 and other inflammatory factors, is induced by elevated ROS production (Siwik et al., 2008; Yuan et al., 2010). This supports the findings that Hcy treatment results in an M1 macrophage phenotype through EMMPRIN, leading to increased ROS production.

M1 macrophages are known to fight infections, cause neurotoxicity and exacerbate atherosclerosis (El Hadri et al., 2012; Kigerl et al., 2009; Tavakoli & Asmis, 2012). These tasks are accomplished through the release of pro-

inflammatory cytokines and MMP-9 as well as increased reactive nitrogen and oxygen species production (de Pinho et al., 2014; Nishi et al., 2008; Sica & Mantovani, 2012; Weisser et al., 2013; Woo et al., 2004). This study further validated the Hcy induced shift towards the M1 phenotype by demonstrating significantly increased ROS production in Hcy treated murine macrophages through a DCFDA assay. Furthermore, it was demonstrated that inhibition of EMMPRIN led to a significant reduction in ROS production in and that the effects of HHcy on ROS production were completely mitigated when EMMPRIN was blocked. These findings agree with a study by El Hadri et al., which demonstrates a predominant population of M1 macrophages in severe atherosclerotic lesions of ApoE2.Ki mice (El Hadri et al., 2012) that are reliant on ROS production. These mice were challenged with lipopolysaccharide (a known inducer of M1) for 5 weeks, causing them to develop atherosclerotic lesions. However, when treated with thioredoxin-1, an anti-oxidative protein, the macrophages in the lesions shifted predominantly to the M2 phenotype and the area of aortic lesion was significantly reduced. The present study, which suggests that CBS^{+/-} gastrocnemius muscle has a greater M1 macrophage population and that the number is reduced by exercise, is further supported by the concept that anti-oxidative proteins cause a shift in macrophage phenotype towards M2. Exercise is known to increase cellular anti-oxidative capacity, which in turn helps to reduce muscular inflammation (Vincent et al., 2006). Decreased levels of muscular inflammation with exercise in CBS ^{+/-} mice would explain the observed reduction in M1 macrophage content in the CBS ^{+/-} skeletal muscle

after training. Although there was an observed decreased concentration of M1 macrophages in CBS +/- mice with exercise, it is not surprising that there was not a significant reduction in overall macrophage presence (F4/80). It has been documented that macrophages are capable of shifting phenotype within the skeletal muscle, depending on the environment (Arnold et al., 2007; Chazaud et al., 2009). Therefore, it is very plausible that the present M1 macrophages shifted into an M2 phenotype or just became relatively inactive. Further research is clearly needed to determine the changes occurring with intramuscular macrophage populations with exercise and HHcy.

In summary, the present study demonstrates that Hcy causes murine macrophages to shift into a classically activated M1 phenotype as determined by CD40 expression. This stimulates EMMPRIN and MMP-9 synthesis as well as increased ROS production. Furthermore, it was found that inhibition of EMMPRIN leads to decreased CD40 in these cells, indicating a shift away from the M1 phenotype. Moreover, it is apparent that HHcy causes a greater M1 macrophage population in the skeletal muscle and that this population shifts to a different phenotype with exercise intervention. Altogether, these results suggest that Hcy promotes an M1 macrophage phenotype through an EMMPRIN mediated pathway and that exercise can mitigate the inflammatory state associated with HHcy and M1 macrophages.

CHAPTER VI

SUMMARY, CONCLUSION AND FUTURE DIRECTIONS

Hyperhomocysteinemia has been widely implicated in the development of cardiovascular diseases such as hypertension, atherosclerosis, heart failure and diabetes (Bortolotto et al., 1999; Signorello et al., 2007; Sutton-Tyrrell et al., 1997; Wiernicki et al., 2011; Zheng et al., 2013). Elevated levels of inflammation, including increased MMP-9 and ROS production are hallmarks of HHcy (Lee et al., 2012; Signorello et al., 2007; Tyagi et al., 2009). Clinical studies of patients with genetic defects that cause HHcy, such as MTHFR or CBS deficiency, display decreased bodyweight and skeletal muscle mass (Kalra et al., 1985; Kanwar et al., 1976). Research has demonstrated that HHcy translates to impaired muscular strength and elicits functional limitations in humans and animals (Swart et al., 2013; Veeranki et al., 2015). With this knowledge, there is sufficient evidence that suggests that HHcy may induce chronic inflammation in skeletal muscle leading to muscular dysfunction. However, the molecular changes and mechanisms associated with this are still very unclear. Furthermore, it has been demonstrated that exercise is capable of reducing plasma Hcy levels (Neuman et al., 2013; Randeva et al., 2002). Therefore, exercise would serve as a promising intervention for individuals with HHcy and HHcy related pathologies.

In the first set of experiments it was found that HHcy led to decreased body mass and skeletal muscle perfusion in CBS +/- mice. It was found that the skeletal muscle of these mice have significantly higher collagen deposition in the skeletal muscle, indicating fibrosis, as well as increased levels of the growth inhibiting factor, myostatin. This study showed that HHcy led to inflammation of the skeletal muscle and that the remodeling likely occurs through elevated MMP-9 levels via EMMPRIN induction. Interestingly, our CBS/MMP-9 double KO mouse model did not display any of the pathologies that are apparent in CBS +/- mice, indicating that HHcy elicits most of its effects through MMP-9. Moreover, this study demonstrates that exercise training can completely mitigate most of these physical and molecular changes found in CBS +/- mice.

In the second set of experiments, it was found that treatment of macrophages with Hcy caused differentiation into a classically activated, M1 phenotype, which is associated with increased MMP-9 and ROS production. It appears that HHcy induction of the M1 phenotype is EMMPRIN dependent, since EMMPRIN induction prevented the M1 switch. Furthermore, this study demonstrated that CBS +/- mice have a greater concentration of macrophages in their skeletal muscle and that a great number of these macrophages were of the M1 phenotype. Although exercise did not significantly decrease the total macrophage concentration within the skeletal muscle, it was able to significantly reduce the number of M1 macrophages present, indicating a lower state of inflammation and adverse remodeling. Although the data is significant and conclusive, our study was limited. A longer exercise protocol as well as different

modalities of exercise would help make greater comparisons for this study. Also, macrophages were not isolated from the skeletal muscle, so limited conclusions could be made based on the IHC for co-localization of F4/80 and TNF- α for indication of M1 macrophages. Isolation of macrophages from the skeletal muscle could lead to more conclusive data.

Future Directions:

Skeletal muscle remodeling involves a great number of cell types and secreted factors that regulate growth, repair and fibrosis of the skeletal muscle ECM. The primary focus of this study was to determine if HHcy promotes skeletal muscle fibrosis through induction of MMP-9 and the M1 macrophage phenotype and whether or not exercise can mitigate the effects of HHcy. Although this study was able to demonstrate these effects, there are many other factors and signaling pathways that need to be determined to devise the mechanism through which HHcy causes skeletal muscle dysfunction.

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CURRICULUM VITA

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Sellersburg, IN 47172

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Education

University of Louisville – Louisville, KY

Doctor of Philosophy August 2015

Physiology and Biophysics

Proposed Graduation Date: August 2015

3.87/4.0 Current Graduate GPA

University of Louisville - Louisville, KY

Masters of Science

Physiology and Biophysics

3.87/4.0 GPA

University of Louisville - Louisville, KY

Masters of Science August 2010

Exercise Physiology

3.84/4.0 GPA

Master's Thesis – "Systemic and strength effects: experimental concentrations of topical menthol"

Purdue University- West Lafayette, IN

Bachelors of Liberal Arts May 2008

Health and Fitness Major/ Health Promotion Major

3.2/4.0 Major

Significant Coursework: Advanced Biochemistry, Cell Biology, Advanced Endocrinology, Systemic Physiology I and II, Advanced Human Physiology, Lab Methods in Exercise Physiology, Advanced Exercise Physiology, Exercise Physiology/Human Bioenergetics, Graduate Statistics, Anatomy and Physiology, Human Diseases and Disorders, Motor Development, Biomechanics, Worksite Health Promotion, Health and Fitness Program Planning, Health and Fitness Program Management, Health and Fitness Research Methods, Substance abuse and Human Health, Epidemiology, Health and Fitness Assessments and Exercise Prescription, Health Behavior Change, Health Psychology, Stress and

Human Health, Sexuality and Human Health, Women's Health, Essentials of Nutrition, Communications, Spanish to level 4

Related Skills and Techniques: Real Time PCR, RNA Purification, Immunohistochemistry, Immunocytochemistry, Trichrome collagen staining, Flow Cytometry, HPLC, Semi-quantitative PCR, Western Blotting, Cell Culture and Treatment, Animal handling and surgical procedures, Reactive Oxygen Species production Assays, Laser Doppler imaging, Ultrasound Doppler for Blood Flow Analysis, Exercise training in humans and animals, VO2 Max and Submax testing, Operation of Biodex Dynamometer, Multiple methods of Body Composition Analysis including Hydrostatic Weighing Analysis, Lactate Threshold Testing, Familiar with SPSS statistical software and Microsoft Office.

Professional Experience

IPIBS PhD Fellowship, University of Louisville

Louisville, KY 08/10 – Present

Conduct research involving specific molecular targets of cancer

Complete relevant coursework towards obtaining PhD degree

Graduate Research/Teaching Assistant, University of Louisville

Louisville, KY 08/08 – 08/10

Data collection and analysis

Protocol development and IRB submission

Subject recruiting and scheduling

Anatomy and Physiology course assistance and instruction

Graduate Lab Methods course assistant

Fitness Consultant/Personal Trainer, Riverside Health Care

Bourbonnais, IL 05/08 – 08/08

Perform Fitness Assessments

Give equipment orientations

Develop various fitness programs

Assist with membership and sales

Personal training

Assistant Manager and Personal Trainer, Levee Plaza Health and Fitness

West Lafayette, IN 05/06 – 05/08

Help patrons with membership needs and questions.

Maintained a clean environment for patrons to workout in.

Supervise employees during the evening hours.

Educate patrons about proper equipment usage

Personal Trainer/Fitness Advisor, Purdue University Colby Fitness Center

West Lafayette, IN 01/06-08/07

Interacted with clients by providing a solid workout schedule and diet.

Instructed the patrons with proper form and technique of exercises.

Educated clients about the importance of lifetime physical activity.

Perform standard fitness tests on clients to determine health status.

Student Assistant, Purdue University WorkLife Programs

West Lafayette, IN 02/07 – 07/07

Assisted with overall organization of a program titled Targeted

Communication.

Helped organize mass mailings for the Targeted Communication program.

Helped to lead patrons through a walking program.

Assisted in numerous other office related tasks

Head Fitness Center Supervisor, Purdue University Colby Fitness Center

West Lafayette, IN 01/05 – 11/05

Organized the fitness center staff duties and schedules

Performed duties such as Payroll and kept track of the number of patrons.

Personal Trainer and Supervisor, Harrison Barbell Fitness Center

Harrison, OH 05/05 – 08/05

Interacted with clients by providing a solid workout schedule and diet.

Instructed the patrons with proper form and technique of exercises.

Assisted in the organization of the fitness center and memberships.

Teaching Experience

Guest Lecturer in Exercise Physiology, Hanover College.

Hanover, IN 10/23/14 and 10/3/13

I provided guest instruction to an undergraduate pathophysiology class. In October of 2014 the topic of discussion was inflammation induced pathologies of skeletal muscle and vascular dysfunction. In October of 2013 I provided a guest lecture about iron metabolism and reactive oxygen production.

**Teaching Assistant for Undergraduate Anatomy and Physiology,
University of Louisville**

Louisville, KY 08/09 - 05/10

My role as the teaching assistant was to grade assignments and proctor examinations. During this time I gave several presentations on endocrinology.

**Teaching Assistant for First Year Graduate Exercise Physiology
Laboratory Methods Course, University of Louisville**

Louisville, KY 08/09 – 12/09

This course was divided into two parts: a lecture section and a laboratory section. I directly provided instruction for the laboratory section demonstrating proper protocols and techniques utilized in the exercise physiology laboratory.

Publications

Winchester, L., Veeranki, S., & Tyagi, S.C., Homocystiene Elicits and M1 Phenotype in Murine Macrophages Through an EMMPRIN Mediated Pathway. *Canadian Journal of Physiology and Pharmacology*. 2015, Mar 31:1-8.

Givvimani, S., Kundu, S., Pushpakumar, S., Doyle, V., Narayanan, N.,
Winchester, L., Veeranki, S., & Metreveli, N. Hyperhomocysteinemia a Missing Link to Dysfunctional HDL via Paraoxanase-1. *Canadian Journal of Physiology and Pharmacology*. 2015 Apr 15:1-9.

Veeranki, S., **Winchester, L.**, & Tyagi, S.C. Hyperhomocysteinemia (HHcy) associated skeletal muscle weakness involves mitochondrial

dysfunction and epigenetic modifications. *Biochimica et Biophysica Acta*. 2015 May;1852(5):732-41.

Winchester, L., Veeranki, S., Givvimani, S., & Tyagi, S.C. Exercise mitigates the adverse effects of hyperhomocysteinemia on macrophages, MMP-9, skeletal muscle, and white adipocytes. *Canadian Journal of Physiology and Pharmacology*. 2014 Jul;92(7):575-82

Lambert, C.P., **Winchester, L.,** Jacks, D.A., & Nader, P.A. Sex Differences in Time to Fatigue at 100% VO₂ peak When Normalized for Fat Free Mass. *Research in Sports Medicine: An International Journal*. 2013; 21(1): 78-89.

Topp, R., **Winchester, L. J.,** Sannes, S. H., Mink, A. M., Kaufman, J. S., & Jacks, D. E. A Comparison of Ice with 3.5% Menthol Gel on Blood Flow and Muscle Strength of the Lower Arm. *Journal of Sport Rehabilitation*. 2011; 20: 355-366.

Topp, R., **Winchester, L. J,** Schilero, J. & Jacks, D. E. Effect of Topical Menthol on Ipsilateral and Contralateral Superficial Blood Flow Following a Bout of Maximum Voluntary Muscle Contraction. *International Journal of Sports Physical Therapy*, 2011 Jun: 6(2): 83-91.

Poster Presentations

Exercise Mitigates the Deleterious Effects of Hyperhomocysteinemia on Muscular Function and Inflammation. **Lee J. Winchester**, MS, Sudhakar Veeranki, PhD, Sathnur Pushpakumar, MD, PhD, Srikanth Givvimani, MD, PhD, and Suresh C. Tyagi, PhD. September 2014. ACSM Integrative Physiology of Exercise Conference. Miami Beach, FL.

Reversing Increased M1/M2 Macrophage Ratio in Hyperhomocysteinemic Skeletal Muscle With Exercise. **Lee J Winchester**, MS, Sudhakar Veeranki, PhD, Sathnur Pushpakumar, M.D., Ph.D., and Suresh C. Tyagi, PhD. April 2014. Experimental Biology Conference. San Diego, CA.

Reversing Hyperhomocysteinemia Induced Skeletal Muscle Myopathy With Regular Exercise. **Lee J Winchester**, Sudhakar Veeranki and Suresh C. Tyagi. March 2014. Kentucky Physiological Society Annual Meeting. Louisville, KY

Induction of Ferritin by Small Molecules may Have Cytoprotective Effects. **Winchester, L.**, Trent, J., Lamont, G., & Eaton, J.W. October 2013. James Graham Brown Cancer Center Retreat, University of Louisville Louisville, KY.

Activities and Honors

Graduate Student Council Representative

In 2010 I served as the student representative for the Health and Sports Sciences Department on the Graduate Student Council at the University of Louisville. Meetings discuss issues related to graduate student activities, primarily travel funds. In February 2009, I organized the graduate student funding procedures for the Exercise Physiology department's trip to the Southeast ACSM conference.

Student Lab Assistant, Purdue University Wastl Exercise Physiology Lab

During my undergraduate career I assisted with research in the Wastl Exercise Physiology lab in the basement of Lambert Hall, primarily dealing with carbohydrate studies. I assisted in preparation procedures as well as various techniques during VO₂ max testing, including blood collection and analysis.

Professional Memberships

American Physiological Society

Graduate Student Member since 2013

American College of Sports Medicine

Graduate Student Member since 2008