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UNIVERSITY OF NORTHERN COLORADO

Greeley, CO

The Graduate School

THE EFFECTS OF CONTRACTION ON SKELETAL MUSCLE EXPRESSION OF INTERLEUKIN-6

A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of
Master of Science

Drew Huber

The College of Natural and Health Sciences
School of Sport and Exercise Science
Exercise Physiology

May 2022

This Thesis by: Drew Huber

Entitled: *The Effects of Contraction on Skeletal Muscle Expression of Interleukin-6*

Has been approved as meeting the requirement for the Degree of Master of Science in College of Natural and Health Science in School of Sport and Exercise Science, Program of Exercise Physiology

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ABSTRACT

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Interleukin-6 (IL6) is a pleiotropic cytokine secreted by a wide array of cells in response to different stimuli. The immune and inflammatory functions of IL6 have been widely studied, and it is well documented that IL6 plays a critical role in immune function and the inflammatory response to pathogens and damage to the organism. Interleukin-6 has also been implicated in the pathogenesis of a wide array of diseases and high circulating levels of IL6 are used as a clinical marker for many. However, recent work has found IL6 to function as a myokine in response to exercise involving muscular contractions. Myokines are proteins and cytokines released by skeletal muscle in response to contraction that have been shown to have a beneficial effect on metabolism, muscle hypertrophy, angiogenesis, and decreasing levels of chronic inflammation. These contradictory roles of IL6 make studying the potential benefits it has in response to exercise of profound importance. However, methods used to discover how and where skeletal muscle is secreting IL6 are not definitive and further research is needed to help understand muscle derived IL6. **Purpose:** To study whether IL6 is expressed in skeletal muscle from rats using an *ex vivo* model to rule out expression by other cells during exercise. **Methods**: Male Sprague-Dawley rats were sacrificed and had their soleus (SOL) and extensor digitorum longus (EDL) excised. Left-sided muscles were flash frozen and stored as a sedentary control. Right sided muscles were stimulated to contract until fatigued. **Results:** There were no significant differences between control and exercised soleus muscles IL6 ($p = 0.328$) or control and exercised extensor digitorum longus muscles IL6 ($p = 0.41$). **Conclusion:** There were no

significant differences between groups, but a trend was noted that exercised muscles expressed less IL6 than their sedentary counterparts. This is thought to be due to muscle secretion of IL6 from the working skeletal muscle. These findings warrant further study.

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CHAPTER I

INTRODUCTION

Interleukin-6 (IL6) is a pleiotropic cytokine that is synthesized and secreted by a wide array of cells and tissues in response to different stimuli. Interleukin-6 was first discovered for its regulatory functions in the immune system, specifically the acute phase response (Carson & Baltgalvis, 2010). Interleukin-6 was found afterwards to exert its effects on a wide array of cells and tissues depending on the stimulus.

The recent discovery that skeletal muscle acts as an endocrine organ and secretes its own cytokines and proteins to facilitate crosstalk between tissues presents a whole new field of study regarding exercise and adaptations based on tissue crosstalk. Originally thought of as solely a proinflammatory cytokine, IL6's recent role as a muscle myokine has revealed both redundant and confounding functions (Pal et al., 2014). While IL6 is often associated with the pathogenesis of various diseases, the new possibly anti-inflammatory roles for IL6 need to be further evaluated for potentially non-pharmacological protocols for a wide array of chronic diseases. In response to exercise, IL6 levels are found to increase more quickly than any other cytokine with levels increasing up to 100-fold immediately after exercise (Kaniganti & Majumdar, 2019). The mode and intensity of exercise are thought to be the factors regulating the amount of IL6 secreted from skeletal muscle into the blood stream (Pedersen, 2013).

As a myokine, IL6 was found to have beneficial effects on metabolism, muscle hypertrophy, immunity, and insulin sensitivity (Pedersen & Fischer, 2007; Serrano et al., 2008). Exercise-induced skeletal muscle secretion of IL6 has been theorized to increase insulin

sensitivity and allow the working skeletal muscle to uptake more glucose from the blood stream and increase glycogenolysis and gluconeogenesis pathways in the liver (Covington et al., 2016; Garneau & Aguer, 2019).

Muscle hypertrophy is thought to be mediated by IL6 via its role in the activation, proliferation, and migration of the adult stem cells called satellite cells (SCs) (Serrano et al., 2008). Satellite cells promote muscle hypertrophy by proliferating, dividing, and fusing to already existing myofibers based on what the working skeletal muscle needs for repair (Toth et al., 2011). Furthermore, when IL6 is secreted from muscle contractions during exercise, there seems to be a decrease in inflammatory cytokines such as tumor necrosis factor α (TNFα) and an elevation in the secretion of anti-inflammatory cytokines such as interleukin-10 (IL10) and interleukin-1 receptor antagonist (IL1RA) (Schnyder & Handschin, 2015).

Moderate exercise has been shown to have protective effects and increase immunity to intracellular infections which can be stimulated by the proinflammatory response (Kaniganti & Majumdar, 2019). While high intensity exercise has been shown to potentially increase vulnerability to infections, it also encourages the anti-inflammatory response in order to decrease post-exercise muscle tissue damage and inflammation (Kaniganti & Majumdar, 2019). Interleukin-6 is thought to be a key regulator in the anti-inflammatory response and is theorized to be transcribed in cells due to the formation of reactive oxygen species (ROS), impaired glucose availability, and altered muscle calcium levels (Kaniganti & Majumdar, 2019).

The methodology researchers used to find that IL6 is secreted by skeletal muscle and not another tissue must be investigated. Immune cells have been shown to secrete IL6 in response to inflammatory stimuli, pathogens, or damage to the organism. In an *in vivo* model, we cannot be sure if the IL6 is being secreted by skeletal muscle or other adjacent cells and taken up by

muscles to promote systemic changes. Previous studies have theorized that skeletal muscle is synthesizing and secreting IL6 in response to contractile stimuli by measuring concentrations in the plasma and whether there had been infiltration by blood mononuclear cells (BMNCs) like monocytes and macrophages into the muscle that secrete IL6 (Ostrowski et al., 1998; Van Hall et al., 2003). An *ex vivo* model of muscular contraction and analysis of IL6 levels should therefore be undertaken to determine if the rapid increase of exercise induced IL6 is solely due to synthesis and secretion of skeletal muscle.

Interleukin-6 is a pleiotropic cytokine with various, sometimes contradictory functions in the body. Exercise-induced expression of IL6 in skeletal muscle may have beneficial metabolic effects and improve skeletal muscle health, thus helping people with a wide range of metabolic diseases and disorders.

1.1 Statement of Purpose

The primary focus of this study was to determine if there is a significant increase in skeletal muscle expression of IL6 in response to exercise using an *ex vivo* muscle contraction model.

1.2 Research Hypothesis

H1 Rat skeletal muscle will express significantly more IL6 in response to muscular contraction compared to the control groups.

CHAPTER II

REVIEW OF LITERATURE

2.1 Background

Chronic, noncommunicable illnesses continue to impact the world increasingly. By 2020, non-communicable diseases were expected to account or 70% of deaths and 60% of the disease burden (Pedersen, 2011). Being overweight and obese result in more deaths in recent years than malnutrition (Li et al., 2017). As of 2014, almost 39% of adults worldwide over the age of 18 were overweight and 13% were obese (Li et al., 2017).

Type 2 Diabetes Mellitus (T2D) affects 8.5% of the adult population with over 422 million people worldwide according to the World Health Organization (WHO) (Garneau & Aguer, 2019). The principle risk factor for T2D is excess body weight (Garneau & Aguer, 2019). However, Pedersen (2011) noted that there is a J shape association between body mass index (BMI) and mortality, meaning that both high and low BMIs are associated with early death and mortality. The risk factors for having a very low BMI are speculated to be due to a low amount of lean body mass compared to fat mass rather than simply a low percentage of fat mass (Pedersen, 2011).

Independent of BMI, physical inactivity is considered a risk factor for all-cause mortality (Lavie et al., 2019; Pedersen, 2011). Physical inactivity is defined as energy expenditure <1.0 metabolic equivalent of a task (MET) while in a seated, reclined, or lying posture (Lavie et al., 2019). Sedentary behaviors and physical inactivity are considered modifiable risk factors for cardiovascular disease (CVD) (Lavie et al., 2019). The Center for Disease Control (CDC) has

designated physical inactivity as an actual cause of chronic illnesses. Worldwide, 31% of people are considered physically inactive while 50% of adults in the United States are considered physically inactive. (Furman et al., 2019) In fact, sedentary populations have life expectancies on average 5 years shorter than their physically active counterparts (Pedersen, 2011). Physical inactivity is the 4th leading cause of death in the world according to the WHO (Gomarasca et al., 2020). Physical inactivity in 2008 accounted for over 5.3 million premature global deaths (Lavie et al., 2019). Researchers theorize that eliminating physical inactivity could result in an increased lifespan of 0.68 years (Lavie et al., 2019). Physical inactivity has been shown to have a dose-response relationship with sitting time and all-cause mortality (Lavie et al., 2019). In a study on 8,800 Australians over a 6.6-year timeframe, researchers found that those who watched TV $\geq$ 2 hours/day increased their risk for all-cause and CVD mortality by 45% and those that watched TV $\geq$ 4 hours/day had an 80% increase for all-cause and CVD mortality (Lavie et al., 2019).

The current recommendation for physical activity is at least 150 minutes of moderate to vigorous exercise per week (Gomarasca et al., 2020; Lavie et al., 2019). Along with this, the American Diabetes Association recommends not having prolonged sedentary behavior but to break it up with short bursts of light-intensity physical activity (Lavie et al., 2019). However, a large majority of the United States and the world still present with low physical activity levels and increased sedentary behavior (Lavie et al., 2019). This lack of physical activity is thought to be from a number of factors like the increased adoption of the Western lifestyle which tends to promote greater sedentary time, lower participation in active transport, and more time spent in leisure with less purposeful physical activity (Lavie et al., 2019).

Pedersen (2011) theorized there is a phenomenon called the diseasome of physical inactivity. The diseasome of physical inactivity speculates that a lack of physical activity alone

would increase the risk for developing chronic illnesses like Type II Diabetes (T2D), CVD, colon cancer, postmenopausal breast cancer, dementia, and depression (Pedersen, 2011). Physical activity in previous research has been found to decrease the risk of coronary artery disease (CAD) by 6%, T2D by 7%, breast cancer by 10%, and colon cancer by 10% (Lavie et al., 2019). Indeed, decreasing physical activity from 10,000 steps/day to 5,000 steps/day resulted in decreased blood flow in participants' popliteal artery and decreased endothelial cell function (Lavie et al., 2019). There were increases in several markers of endothelial apoptosis in the decreased physical activity group like CD31+/CD42b- (Lavie et al., 2019)

2.2 Chronic Inflammation and Disease Pathogenesis

Diseases that can result from physical inactivity and sedentary behavior like T2D, CVD, and various cancers also show systemic, chronic low-level inflammation (Manole et al., 2018; Pedersen, 2011). Systemic, chronic inflammation (SCI) is the low-grade persistent activation of the inflammatory response acting over an extended period of time (Furman et al., 2019). Inflammation is the process by which the immune system and non-immune cells protect the body from foreign pathogens like viruses, bacteria, toxins, and other infections (Furman et al., 2019). The role of the inflammatory response is to promote the repair of tissue and its recovery. The level of inflammation, whether it is systemic, local, metabolic, or involves neuroendocrine changes depends on the degree and extent of the response to a foreign or domestic pathogen (Furman et al., 2019). During the inflammatory response there can be changes that occur to conserve energy and allocate more nutrients for the function of the immune system (Furman et al., 2019). Inflammation can cause behaviors known as "sick behaviors" due to its processes of eliminating pathogens (Furman et al., 2019). Symptoms of "sick behaviors" due to inflammation

can include sadness, fatigue, reduced libido, reduced food intake, altered sleep, increased blood pressure, insulin resistance, and dyslipidemia (Furman et al., 2019; Gomarasca et al., 2020).

Normally the inflammatory response should cause an upregulation of inflammation and then the symptoms should pass when the threat has passed (Furman et al., 2019). Inflammation can be caused by a number of factors, not just pathogens. Inflammation can be triggered by certain social, psychological, environmental, and biological factors (Furman et al., 2019). Acute inflammation is typically caused during periods of infections where pathogens are recognized by pathogen-associated molecular patterns (PAMPs) on the cells of the innate immune system (Furman et al., 2019). However, inflammation can also be recognized during periods of damage to tissues via damage-associated molecular patterns (DAMPs) receptors (Furman et al., 2019). During SCI that is punctuated by low-level inflammation, the primary trigger is the DAMPs in the absence of a pathogen causing harm to the body (Furman et al., 2019). Systemic, chronic inflammation is categorized as low-grade persistent inflammation that over time can cause collateral damage to many different tissues in the body (Daou, 2020; Furman et al., 2019).

Many different non-communicable diseases have their pathogenesis in part due to chronic SCI which allows them to thrive at the expense of the organism (Furman et al., 2019; Pedersen, 2011; Pedersen & Fischer, 2007). Certain cancers, T2D, Rheumatoid Arthritis (RA), cancer cachexia, and sarcopenia have been shown to have the common theme of SCI driving their pathogenesis (Carson & Baltgalvis, 2010; Gomarasca et al., 2020).

Currently, there is no definitive measure for SCI (Furman et al., 2019). Many of the increased markers for SCI appear to be due to the normal process of aging (Furman et al., 2019). As individuals get older there appears to be greater levels of inflammation making detecting SCI hard to determine if it is from normal aging or the pathogenesis of chronic, non-communicable

diseases (Furman et al., 2019). At the time of this writing, there is no definitive protocol for determining SCI, but newer techniques are being developed such as profiling of individual's whole-blood gene expression for cytokines, chemokines, CD8+ T cells, monocytes, natural killer cells (NK), B cells, and CD4+ T cells (Furman et al., 2019).

2.3 Excess Adipose Tissue and Systemic Chronic Inflammation

Chronic low-level inflammation is a known feature in obese individuals and those who are physically inactive (Garneau & Aguer, 2019; Pedersen, 2011). Obese individuals with an excess of white adipose tissue (WAT) are known to exhibit chronic low-level inflammation (Garneau & Aguer, 2019; Gomarasca et al., 2020). Adipose tissue is now known not just to be an energy storage organ but a major endocrine organ (Li et al., 2017; Lutosławska, 2012).

Adipose tissue is known to secrete proteins called adipokines involved in maintaining homeostasis between tissues and adipokines help to facilitate crosstalk between adipose and other tissues (Gomarasca et al., 2020; Li et al., 2017). Often with excess WAT, there is an increased secretion of proinflammatory adipokines like tumor necrosis factor alpha (TNF-a), interleukin-6 (IL6), C-reactive protein (CRP), and monocyte chemoattractant protein 1 (MCP1) (Gomarasca et al., 2020; Li et al., 2017). These proinflammatory adipokines can predispose individuals to develop insulin resistance, dyslipidemia, atherosclerosis, skeletal muscle wasting, and other non-communicable illnesses (Gomarasca et al., 2020; Pedersen, 2013). The increased secretion of adipokines from adipose tissue is thought to be through the accumulation of immune cells, specifically proinflammatory macrophages releasing their own cytokines and causing adipose tissue to release even more proinflammatory cytokines driving SCI when there is excess hypertrophy of an individual's adipose tissue (Gomarasca et al., 2020; Pal et al., 2014). This process does appear to be blunted in individuals with less adipose tissue and more lean tissue

mass (Pal et al., 2014). Specifically, leaner individuals seem to have a higher expression of M2 anti-inflammatory macrophages compared to those with excess adipose tissue (Pal et al., 2014).

Skeletal muscle is a major target of adipokines for the crosstalk between skeletal muscle and adipose tissue (Li et al., 2017). The crosstalk between adipose tissue and skeletal muscle has major metabolic consequences and can implicate energy homeostasis for the entire body (Li et al., 2017). The crosstalk between adipose tissue and skeletal muscle might play a significant role in the rate and/or extent of myogenesis, adipogenesis, protein turnover, and lipogenesis/lipolysis (Li et al., 2017). This crosstalk may also play a key role in the modulation of body composition (Li et al., 2017).

Skeletal muscle has also been recently found to have endocrine functions that affect total body homeostasis (Pedersen, 2011). Skeletal muscle has been found to secrete its own growth factors, cytokines, and other proteins in response to different stimuli that act in autocrine, paracrine, and endocrine signaling ways (Li et al., 2017; Lutosławska, 2012; Pedersen, 2011). When in response to stimuli like exercise and training, skeletal muscle has been shown to increase secretion and expression of these proteins (Garneau & Aguer, 2019; Li et al., 2017; Schnyder & Handschin, 2015).

2.4 Exercise, Physical Activity, and Systemic Chronic Inflammation

Physical activity and exercise have many health benefits in regard to all-cause mortality and the development of chronic, non-communicable illness and SCI (Furman et al., 2019; Lavie, et al., 2019; Saeidifard et al., 2020). Physical inactivity on the other hand has been previously identified as a risk factor for many chronic illnesses and SCI. Physical activity and exercise are known to reduce SCI, promote insulin sensitivity, and reduce risks of developing a wide array of metabolic and cardiovascular related diseases (Lavie et al., 2019). Physical activity and exercise

training have been shown to attenuate the symptoms and pathogenesis of a multitude of diseases that are expressed by chronic physical inactivity (Lavie et al., 2019). Increased cardiorespiratory fitness (CRF) is associated with a reduction in the prevalence of many CVD risk factors such as hypertension, obesity, metabolic syndrome, and T2D (Lavie et al., 2019). A recent meta-analysis showed that for every 1 MET increase in CRF there was a 13%-15% reduction in CVD and all-cause mortality (Lavie et al.,) making CRF an integral part in reducing the detrimental effects of being sedentary and/or physically inactive.

Cardiorespiratory fitness represents the functioning of multiple organ systems to effectively transport oxygen from the air to the mitochondria of working skeletal muscle to meet the necessary energy demands of the activity and can make the body more efficient at clearing metabolic byproducts (Lavie et al., 2019). Experts agree that doing some physical activity/exercise in terms of dosing is better than doing nothing at all (Lavie et al., 2019). There appears to be a dose response relationship between aerobic physical activity and mortality with some mortalities being reduced with only 15 min/day of moderate physical activity (Lavie et al., 2019). In fact, active runners over a span of 15 years had a 30%-45% reduction in their risk of developing CVD mortality and had increased lifespans of 3-4.1 years (Lavie et al., 2019).

The benefits of resistance training compared to aerobic training in terms of general health are unclear and continue to be studied. Resistance training constitutes the utilization of free weights or machines up to and exceeding 65% of a participant's 1 repetition maximum (1RM) (Saeidifard et al., 2020). Resistance training can be further divided into dynamic and isometric resistance training. Dynamic resistance training consists of moving weights and/or machines through concentric (shortening) and eccentric (lengthening) muscle contractions (Saeidifard et

al., 2020). Isometric resistance training consists of static exertion on a load that results in no change in the muscle length (Saeidifard et al., 2020).

Relatively short periods of resistance training could lead to improvements in CVD risk factors like insulin resistance, glucose and lipid metabolism, and impaired endothelial function with reduced sympathetic neural activity (Saeidifard et al., 2020). Those who participate in resistance training have been shown to have a decrease in their resting systolic and diastolic blood pressure (Saeidifard et al., 2020). Resistance training has also been shown to decrease the risk of a myocardial infarction (MI) in participants (Saeidifard et al., 2020). Resistance training has also been shown to improve body composition by the reduction of adipose tissue, specifically visceral adipose, which as explained above can lead to an increase in SCI (Li et al., 2017; Saeidifard et al., 2020). In addition, resistance training increases lean body mass and can prevent and/or slow the development of sarcopenic obesity and age-associated muscle loss (Saeidifard et al., 2020). Resistance training also helps to promote insulin sensitivity and mitochondrial function via increases in the expression of the glucose transporter type 4 (GLUT4) increasing glucose uptake and glycogen synthesis in muscle (Lavie et al., 2019; Saeidifard et al., 2020).

Unlike aerobic training, there is no association with resistance training and telomerase activity, meaning likely there are no anti-aging effects with attributed solely to resistance training (Saeidifard et al., 2020). There was also no association with resistance training and survival for cancer patients although resistance training is speculated to be important for increasing the quality of life (QOL) for these types of patients (Saeidifard et al., 2020). Unlike with aerobic training where there seems to be minimal consequences for doing too much or too little, with resistance training, there appears to be an inverted U-shaped curve for its dosage (Lavie et al.,

2019; Saeidifard et al., 2020). There have been documented adverse effects of too high-intensity training increasing arterial stiffness via increased sympathetic nervous system activity (Saeidifard et al., 2020). Also, the Valsalva maneuver utilized in resistance training by some participants may lead to changes in heart rhythm such as bradycardia or atrial ectopy (Saeidifard et al., 2020).

The current recommendation by researchers is to utilize a combination of resistance training and aerobic training for health benefits, improving body composition, and to reduce the risks of developing CVD, metabolic syndrome, cancer, T2D, dementia, and depression (Daou, 2020; Lavie et al., 2019; Pedersen, 2011; Saeidifard et al., 2020). With the beneficial effects of exercise stated above, it is of paramount importance to understand the factors secreted during exercise, specifically by skeletal muscle that can prevent and treat non-communicable diseases such as the ones discussed above. By understanding factors secreted by skeletal muscle, therapies and protocols can be developed for treatment and prevention of these diseases.

2.5 Skeletal Muscle Overview and the Introduction of Myokines

The human body contains over 600 muscles that can contribute anywhere from 40%-50% of a person's bodyweight and contribute about 40% of an individual's total body proteins (Lightfoot & Cooper, 2016; Schnyder & Handschin, 2015). Skeletal muscles are considered plastic organs in that they change their characteristics based on the stimulus received to a certain degree (Hoffmann & Weigert, 2017; Schnyder & Handschin, 2015). In previous years, skeletal muscle was thought to be only primarily responsible for the generation of power, locomotion, posture, and breathing (Schnyder & Handschin, 2015); however, in recent years, skeletal muscles have been found to have more functions involved in things like shivering, energy storage, and

the ability to secrete their own factors in order to communicate with non-muscle tissues (Schnyder & Handschin, 2015).

This is where the concept of myokines is introduced. Myokines are cytokines which are small glycoproteins released by skeletal muscle during contractile actions (Manole et al., 2018; Pedersen, 2011; Pedersen & Fischer, 2007). Originally, cytokines were found to exert their roles and functions on the regulation of immune cells (Pedersen, 2013). However, it was soon discovered that cytokines were involved in a complex network of communication between various neuroendocrine tissues and immune cells (Pedersen, 2013). Over the last decade, myocytes have been identified as cells with a high secretory capacity like adipocytes (Pedersen, 2013).

Myokine receptors have been found on various non-muscle tissues such as adipose, liver, pancreas, bone, heart, brain, and immune cells (Manole et al., 2018). Myokines provide a mechanism for intercellular communications like differentiation and proliferation to take place (Lightfoot & Cooper, 2016). Myokines were first found to be secreted from skeletal muscle after athletes ran a marathon, and the cytokine interleukin-6 (IL6) was measured before, upon completion, and two hours after the race via muscle biopsy (Ostrowski et al., 1998). IL6 levels had increased from resting 1.5 ± 0.7 pg/ml to 94.4 ± 12.6 pg/ml immediately after the race was completed (Ostrowski et al., 1998). Originally these inflated values were thought to be a result of the inflammatory response via immune cells secreting their cytokines in response to damaged muscle fibers from the prolonged running. However, after analyzing circulating myocytes in the muscle biopsies, there was no detectable IL6 mRNA suggesting the increase in plasma IL6 concentrations was synthesized and secreted by the skeletal muscle (Ostrowski et al., 1998).

After further research, these skeletal muscle derived cytokines were given the term “myokines” (Pedersen, 2011; Pedersen & Fischer, 2007).

Originally it was thought that muscle fibers had to be damaged for myokines to be secreted; however, further research concluded that myokines are secreted due to the contractile actions of muscle fibers (Gomarasca et al., 2020; Guo et al., 2017). Myokines were found to have various metabolic, regulating, and inflammatory functions through autocrine, paracrine, and endocrine signaling (Guo et al., 2017; Pal et al., 2014). In response to exercise, various myokines are either upregulated or downregulated depending on their functions related to skeletal muscle adaptations, metabolic adaptations, and energy sensing capabilities (Manole et al., 2018).

It has been theorized that myokines in response to exercise can exert various inflammatory, metabolic, and immune functions in order to prevent and treat symptoms and diseases that result from various chronic non-communicable diseases making them a possible therapeutic target for various diseases via pharmacological or other non-pharmacological treatments (Daou, 2020; Gomarasca et al., 2020; Manole et al., 2018; Pedersen, 2011).

2.6 Established Myokines and Their Functions

Myokines expressed by skeletal muscles promote many beneficial effects in regard to general health, reducing risk for non-communicable diseases, and regulating various biological functions of tissues and organs (Gomarasca et al., 2020; Manole et al., 2018; Pedersen, 2013). Currently, there are still many different proteins and cytokines that are being assessed as to whether or not they can be classified as myokines. This section will briefly focus on established myokines, their discovery, function, and signaling patterns.

Characteristics and descriptions of proteins that allow them to be in the category of myokines needs to be described. They must first be a cytokine or protein that is produced,

expressed, and secreted by skeletal muscle fibers. Second, they must exert their function in an autocrine, paracrine, or endocrine function. Third, they must balance and counteract the effects of adipokines. Finally, they must mediate protective effects of muscular exercise regarding diseases associated with a physically inactive lifestyle (Pedersen, 2013). Established myokines include the proteins myostatin, leukemia inhibitory factor (LIF), IL6, interleukion-7 (IL7), brain-derived neurotropic factor (BDNF), insulin-like growth Factor 1 (IGF1), and irisin. Other myokines have been identified, but their functions and methods of regulating muscle and total body communication continue to be researched and discovered.

Myostatin is a member of the transformative growth factor beta (TGFβ) family whose main function is the negative regulation of skeletal muscle mass (Lightfoot & Cooper, 2016; Manole et al., 2018). Elevated levels of myostatin are associated with low levels of muscle mass and knockout of the myostatin gene results in doubling of skeletal muscle, browning of WAT, and increases phosphorylation of AMPK (Li et al., 2017). Myostatin is known to suppress satellite cell activation and myoblast proliferation and facilitates a shift from a fast myosin heavy-chain II (MHCII) muscle profile to that of a slower myosin heavy-chain type I (MHCI) during myogenic differentiation (Li et al., 2017). Serum myostatin has been noted to increase with age, partly explaining the loss in muscle mass frequently experienced by elderly populations (Gomarasca et al., 2020).

Leukemia inhibitory factor (LIF) is a recently discovered myokine first found to be secreted from ascites tumor cells (Pedersen, 2013). Initial observations of LIF revealed its functions for terminal differentiation of myeloid leukemic cells, hence its name (Pedersen, 2013). Leukemia inhibitory factor has been found to have a wide array of functions including platelet formation, proliferation of hematopoietic cells, neural survival and formation, muscle

satellite cell proliferation, and acute phase production by hepatocytes (Pedersen, 2013). Leukemia inhibitory factor in terms of being a myokine appears to have more of an autocrine/paracrine method of signaling. Leukemia inhibitory factor induces satellite cell and myoblast proliferation while preventing premature differentiation in these cells by activating the Janus kinase (JAK) 1/signal transducer and activator of transcription (STAT) 1 and STAT3 transcription factors (Pedersen, 2013). The primary receptor for LIF is expressed in satellite cells but not mature muscle fibers contributing to the idea that LIF is involved in the proliferation but not differentiation in satellite cells and myoblasts (Pedersen, 2013).

Interleukin-7 (IL7) is a cytokine required for T cell and B cell development (Pedersen, 2013). Interleukin-7 has previously been linked to hepatic acute-phase response in mice enhancing the numbers of naïve and memory CD4+ and CD8+ T cells (Haugen et al., 2010). Interleukin-7 has been found to be secreted from striated and multinucleated myotubes that also express myosin (Haugen et al., 2010). Interleukin-7 appears to play a role in the proliferation and migration of satellite cells in response to contraction and acts in an autocrine and/or paracrine fashion (Haugen et al., 2010). However, Interleukin-7 has been found to inhibit satellite cell differentiation by altering their migration (Haugen et al., 2010).

Brain-derived neurotropic factor (BDNF) is a neurotropin known to be secreted by the brain, and recently skeletal muscle with functions controlling body mass and energy homeostasis (Pedersen, 2011, 2013). Brain-derived neurotropic factor primarily exerts its functions through the tyrosine kinase receptor, tropomyosin-related kinase (Trk) (Gomarasca et al., 2020; Pedersen, 2013). Brain-derived neurotropic factor in response to exercise has been shown to increase the phosphorylation of AMPK and acetyl-CoA carboxylase enhancing fat oxidation *in vivo* and *in vitro* (Pedersen, 2013). Brain-derived neurotropic factor has also been theorized to have

neuroprotective functions in response to exercise preventing dementia and is hypothesized to contribute to a feeling of “calmness” after an acute bout of exercise (Pedersen, 2013); however, BDNF appears to exact its function in an autocrine/paracrine fashion and 70%-80% of BDNF release is derived from the brain in response to exercise (Gomarasca et al., 2020).

Insulin-like growth factor 1 (IGF1) is a growth hormone essential for normal bone and tissue growth and development (Kwon et al., 2020). Insulin like growth factor 1 is classically known as an anabolic hormone (Gomarasca et al., 2020; Hamrick, 2011). Muscle hypertrophy was found to increase both IGF1 secretion by the liver and by skeletal muscle (Hamrick, 2011). In 2012, IGF1 was officially identified as a myokine (Kwon et al., 2020). Impaired IGF1 has been shown to cause muscular disfunction and poor mitochondrial function in the hippocampus (Kwon et al., 2020). It is thought that muscle hypertrophy leading to bone anabolism are coupled through an IGF1-mediated paracrine mechanism (Hamrick, 2011).

Irisin is a protein that is cleaved from the receptor fibronectin type III containing 5 (FNDC5) on the cell membrane and is involved with changing the phenotype of WAT into a browner phenotype (Pedersen, 2013). There is a 2-fold increase in serum irisin over 10 weeks of exercise in humans and in response to acute exercise (Manole et al., 2018; Pedersen, 2013). Irisin is also known to increase the oxidative capacity of muscle cells by increasing the expression of uncoupling protein 1 (UCP-1) which is involved with mitochondrial biogenesis and heat production leading to fat/weight loss (Guo et al., 2017; Manole et al., 2018; Pedersen, 2013). Irisin has also been shown to improve glucose homeostasis via reactive oxygen species (ROS)-mediated AMPK pathway activation followed by p38/MAPK GLUT4 translocation in differentiated skeletal muscles (Li et al., 2017). Increased irisin is associated with increased muscle hypertrophy, improved muscular strength, and reduced lipid accumulation (Manole et al.,

2018). Irisin and myostatin have been found to be negatively correlated to one another, with myostatin inhibiting myogenesis and irisin promoting myogenesis (Pedersen, 2013).

2.7 Interleukin-6

Interleukin-6 (IL6) belongs to a granulocyte colony-stimulating family factor-like protein family of cytokines (Pal et al., 2014). The IL6 family also comprises Interleukin-11 (IL11), Leukemia Inhibitory Factor (LIF), Oncostatin M (OSM), Ciliary Neurotropic Factor (CNTF), Cardiotropin-1 (CT), and Cardiotropin Like Cytokine (CLC) (Lutosławska, 2012; Pal et al., 2014). Interleukin-6 is located on the chromosome 7p21 in humans and is translated into a 184 amino acid, 4-helix single-chain polypeptide containing a relative molecular mass ranging from 21kD-30kD depending on the cellular source, glycosylation, and method of preparation (Simpson et al., 1997; West, 2019).

Prior to its cloning and discovery, IL6 was thought to be multiple factors responsible for various functions throughout the body and was coined various titles such as interferon-β2, 26K factor, B-cell stimulatory factor 2, hybridoma growth factor, plasmacytoma growth factor, hepatocyte stimulatory factor, hematopoietic factor, and cytotoxic T-cell differentiation factor (Simpson et al., 1997). These names were given based on the various pleiotropic roles IL6 was found to have in the human body depending on the cell it that responds to its signal transduction. These different factors were eventually found to be the same cytokine, IL6.

2.8 Interleukin-6 Receptor and Signaling

When synthesized and secreted by cells, IL6 will exert its functions via its α receptor, interleukin-6 receptor (IL6R) and β receptor, glycoprotein 130 (gp130) in order to transduce its signal from the plasma membrane to the nucleus (Simpson et al., 1997). Interleukin-6 belongs to the family of type 1 cytokine receptors (Choy & Rose-John, 2017). The IL6R will associate with

2 heterodimers of gp130 to form a complex also containing Janus Kinase (JAK) family of tyrosine kinases bound to the intracellular portion of the IL6R/gp130 complex (Choy & Rose-John, 2017). Glycoprotein 130 has no intrinsic kinase domain, hence the constitutive association with the JAK family kinases.

Once IL6 binds to the IL6R/gp130 complex, there is a transphosphorylation of gp130 by the JAK family kinases of proximal tyrosine residues acting as a docking site for molecules containing SH2 homologies (Choy & Rose-John, 2017; Kimura & Kishimoto, 2010). Four distal tyrosine residues are the docking sites for the signal transducer and activator of transcription (STAT) molecules which become phosphorylated by the JAK proteins. These STAT molecules (primarily STAT3 and STAT1 phenotype) will dimerize and translocate into the nucleus where they will activate their target genes such as myc, bcl-2, cyclin-D1, mcp-1, gremlin-1, MMPs, MAPK, PI3K and the Notch pathways (Choy & Rose-John, 2017; West, 2019). The effects and target genes signaled by IL6 depend on the cell type, strength of signaling and physiological context of signaling (West, 2019). Both the IL6R and gp130 dimers are required for IL6 to transduce its signal into the nucleus of cells.

While gp130 is ubiquitously expressed throughout the body, the IL6R is expressed in only a few types of cells such as macrophages, neutrophils, hepatocytes, myocytes, and monocytes (Pal et al., 2014). However, IL6 can exert its functions on a variety of cells that do not contain the membrane IL6R (mIL6R) by way of trans signaling (Carson & Baltgalvis, 2010; Daou, 2020; Simpson et al., 1997). A soluble IL6R (sIL6R) from proteolytic cleavage and gene splicing can bind to a multitude of cells as long as they contain the β receptor gp130 which as previously discussed is ubiquitously expressed (Choy & Rose-John, 2017). In this way the signal from IL6 can be amplified to a multitude of cells that otherwise would not normally respond to

IL6. The process of proteolytic cleavage of mIL6 to form sIL6R has been found to be mediated by immune cells such as neutrophils (Pal et al., 2014).

Classic signaling by IL6 is theorized to exert most of its anti-inflammatory functions, while trans signaling is often associated with the proinflammatory functions of IL6 (Lightfoot & Cooper, 2016). The controversial and sometimes redundant roles of IL6 will be discussed in respect to the distinct functions it exerts on different cells. While more effects of IL6 are still being studied, it is important to understand the roles IL6 plays in immune function, metabolism, and muscle physiology to grasp its changing and contradicting roles.

2.9 Interleukin-6 and Immune Function

Interleukin-6 was first studied for its important roles regulating the immune system and inflammation. (Choy & Rose-John, 2017; Hoene et al., 2013; Kimura & Kishimoto, 2010). However, IL6 has also been implicated in the pathology of various diseases when overexpressed (Velazquez-Salinas et al., 2019). Interleukin-6 is expressed and secreted by a wide variety of immune cells in different contexts depending on infection, damage, or the inflammatory response (Roxburgh & McMillan, 2015).

In response to infection or damage to tissue, immune cells will secrete IL6 and other proinflammatory cytokines in order to promote tissue repair, remodeling, mitogenic signaling, and angiogenic functions (Roxburgh & McMillan, 2015). Interleukin-6 can be synthesized via a number of activated pathways such as MAPK and PI3K, which promotes stromal cells to secrete IL6 (West, 2019). Interleukin-6 is predominately involved in promoting hepatocytes to produce acute phase proteins in response to injury or damage (Tanaka et al., 2014). Interleukin-6 has been found to be the chief stimulator of acute phase response proteins (Gabay, 2006). Acute phase proteins are defined as a set of plasma proteins with concentrations that increase or decrease by

25% in inflammatory conditions (Gabay, 2006). Often acute phase protein levels are used clinically to assess the level of inflammation in the body.

Interleukin-6 is a pivotal player in T-cell mediated immunity (Choy & Rose-John, 2017). In response to viral infections, IL6 has been shown to have essential responsibilities to mount a proper immune response. Production of IL6 in response to viral infections will lead to the differentiation of monocytes into macrophages via regulating the expression of macrophage colony-stimulating factors, lead to the maturation of B-cells into plasma cells via stimulation by interleukin-21 (IL21) and induce the differentiation of naïve CD4+ T-cells into T-helper 17 (Th17) cells (Velazquez-Salinas et al., 2019). CD4+ T-cells are essential regulator of the immune response and inflammatory disease. This differentiation is important for the defense against pathogens at mucosal sites and the defense against fungal and extracellular bacteria (Kimura & Kishimoto, 2010).

Interleukin-6 in cooperation with interleukin-7 (IL7) and interleukin-15 (IL15) can also induce the differentiation and cytolytic capacity of CD8+ cytotoxic T-cells (Velazquez-Salinas et al., 2019). IL6 also suppresses the differentiation of regulatory T-cells (Tregs) allowing a greater activation of the immune system, but without proper restrictions could lead to autoimmunity (Choy & Rose-John, 2017).

Interleukin-6 is a key stimulator of the acute phase response in response to inflammation. Stimulation of hepatocytes in the liver by IL6 in response to damage or pathogen with induce the synthesis and secretion of factors that are hallmarks of inflammation in the clinic (Choy & Rose-John, 2017). These factors include C-reactive protein (CRP), serum amyloid-A, haptoglobin, ferritin, and fibrinogen. During the acute phase response monocytes, macrophages, and

endothelial cells will secrete IL6. This leads to the recruitment of neutrophils through the activation of chemokines and adhesion molecules (Choy & Rose-John, 2017).

Interleukin-6 inhibits the apoptosis of neutrophils allowing them to survive longer. Neutrophils and other immune cells facilitate the shedding of mIL6R to sIL6R as described above to allow cells that do not normally express the mIL6R the ability to respond to the IL6 signal. In this way IL6 can shape the quality of the ensuing immune response (West, 2019). Under normal circumstances, after IL6 has transduced its signal to the nucleus one of the targets of the IL6/JAK/STAT pathway is the synthesis of the suppressor of cytokine signaling (SOCS) to provide negative feedback for IL6 signaling (Tanaka et al., 2014). Suppressor of cytokine signaling 3 is predominately expressed to inhibit IL6 signaling and reduce the inflammatory response (Hoene et al., 2013). However, during times of chronic infections and/or SCI, IL6 may become overexpressed and lead to the pathogenesis of a multitude of diseases and promote further SCI (Narsale & Carson, 2014; Pal et al., 2014). Whether IL6 itself is causing the progression of SCI and the pathogenesis of various diseases is still debated today. Researchers theorize that IL6 may be being secreted as a compensatory mechanism to inhibit other proinflammatory cytokines, but its signal isn't being received correctly due to negative feedback such as via SOCS3 transcription or an inappropriate reaction from the immune system (Gabay, 2006; Haddad et al., 2005; Pal et al., 2014).

2.10 Interleukin-6 and Systemic Chronic Inflammation

Systemic chronic inflammation as discussed previously, has a pathogenesis for a multitude of disease progressions and more than 50% of deaths worldwide are attributed to inflammatory diseases (Furman et al., 2019). Interleukin-6 being a chief regulator of immune function has been found to have a significant role in the progression of chronic systemic

inflammation. Acute inflammation is a limited beneficial response to injury or antigen. Inflammation is characterized as a complex defense mechanism where leukocytes migrate from the vasculature into damaged tissues to destroy the agents that caused injury of infection (Gabay, 2006). IL6 has been implicated in the shift from acute inflammation, having more beneficial functions, to chronic inflammation leading to a multitude of disease pathogenesis, progression of disease, and damage to organs and tissues (Furman et al., 2019; Gabay, 2006).

Acute inflammation is characterized by predominately neutrophils infiltrating damaged tissue and destroying factors causing injury or infection (Gabay, 2006). Acute inflammation can be triggered via recognition of infection on PAMP, or DAMP receptors as discussed previously. At first, this is facilitated by IL6, but as inflammation progresses there is a shift to monocyte recruitment via the IL6-mediated secretion of chemoattractants like monocyte chemoattractant 1 (MCP-1) and adhesion molecules by endothelial cells (Gabay, 2006). Chronic inflammation (inflammation lasting 24-48 hours) shows a shift from neutrophils to predominately monocytes infiltrating the damaged tissues (Gabay, 2006). Interleukin-6 has been implicated in multiple ways to the “switch” from acute inflammation into SCI. The increased serum sIL6R helps to facilitate the shift from neutrophils to monocyte infiltration. Macrophages and monocytes are implicated as crucial sources for proinflammatory cytokine secretion when activated furthering the effects of SCI.

2.11 Interleukin-6 and Autoimmunity

Interleukin-6 was first implicated in autoimmunity (AI) from findings in cardiac myxoma patients. Cardiac myxoma is a benign tumor on the heart. Cardiac myxoma cells in these patients were found to secrete elevated levels of IL6 and these patients often had AI symptoms that went away with surgical removal of the tumor (Ishihara & Hirano, 2002). Other diseases like

rheumatoid arthritis (RA) were also found to be overexpressing IL6 further implicating it with AI (Choy et al., 2002; Ishihara & Hirano, 2002). Further studies have implicated IL6 with the increased B-cell abnormalities like polyclonal B-cell activation, plasmacytosis, and B-cell neoplasia (Ishihara & Hirano, 2002). Interleukin-6 is vital in the shift from acute inflammation into chronic inflammation. In the chronic phase of inflammation there are continuous signals provided from IL6 support the growth and survival of lymphocytes and myeloid cells which increase serum IL6 by secreting it themselves (Ishihara & Hirano, 2002). This provides the basis for the amplification of chronic inflammatory proliferation of these cells.

Interleukin-6 is important for the maintenance and survival of T-cells and through its signaling increases the transcription of the anti-apoptotic proteins bcl-2 and bcl-xL (Ishihara & Hirano, 2002). Persistent overexpression of IL6 in cooperation with transformative growth factor beta (TGFβ) also leads to an imbalance of the Tregs to Th17 cell ratio leading to continued function of the immune system and an increased risk for developing autoimmune disorders like rheumatoid arthritis (Kimura & Kishimoto, 2010). Tregs are a type of regulatory cell responsible for curbing the activity of the immune system in order to prevent AI (Kimura & Kishimoto, 2010; Tanaka et al., 2014). Th17 cells who's maturation is achieved via the synergistic mechanisms of IL6 and TGFβ secrete interleukin-17 (IL17) and are now known to play a crucial role in the induction of AI diseases (Kimura & Kishimoto, 2010).

2.12 Interleukin-6 and Cancer Cachexia

Interleukin-6 has been shown to promote tumor growth and metastasis in a wide array of cancers (Daou, 2020). Cancer cachexia is defined as a 5% decrease in a person's bodyweight over a span of 6 months (Daou, 2020). Cancer cachexia can affect up to 80% of cancer patients and is the primary cause of death for 22%-30% of patients who develop it (Narsale & Carson,

2014). Systemic chronic inflammation is widely thought of as an important regulator of cachexia, and IL6 when overexpressed is a key contributor to the shift from acute inflammation to chronic inflammation with an immune response. The prolonged activation of proliferation, survival, and acute phase response can lead to tumorigenesis and hypermetabolism via the recruitment of the sIL6R by the adaptive immune system (Narsale & Carson, 2014).

Skeletal muscle is the prime target of IL6-mediated cachectic wasting via its downstream targets, namely the JAK/STAT3 pathway (Haddad et al., 2005; Roxburgh & McMillan, 2015). Interleukin-6 overexpression by cells during periods of chronic inflammation similar to levels experienced during exercise over a prolonged period of time induces muscle wasting seen during diseases like cancer cachexia. Haddad et al., (2005) found that infusion of mice with IL6 at similar levels to those seen post-exercise over 14 days had significant atrophy of infused muscles. Over the 14 days, the tibialis anterior muscles of the rats had a 9% decrease in total protein content from basal levels of 168.4 mg ± 8 mg to 152.1 mg ± 7 mg and a 17% decrease in myofibrillar proteins alone (Haddad et al., 2005). However, despite these results, there was no significant changes to the overall body weight of rats indicating that the IL6 infusion had no systemic effects, although the authors theorize this could be due to the relatively low dose and the animal's ability to clear the IL6 infusion from their plasma efficiently.

Interleukin-6 also has a role in the metabolism of bone during periods of SCI. Interleukin-6 contributes to local and systemic bone loss that is associated with multiple pathologies including bone metastases from breast cancers (Gomarasca et al., 2020). Interleukin-6 is thought to cause bone loss through osteoclastogenesis and the differentiation of osteoclasts through the receptor activator of nuclear factor kappa-B ligand (RANKL) causing bone reabsorption (Gomarasca et al., 2020).

There is some debate as to whether the increased systemic levels of plasma IL6 are contributing to muscle wasting seen in cancer cachexia and other diseases, or whether the elevated levels are a compensatory mechanism by which the body is trying to regulate inflammation. Haddad et al. (2005) theorized that the molecule SOCS3 may actually be causing some of the skeletal muscle wasting symptoms seen in inflammatory diseases like cancer cachexia. SOCS3 has been shown not only to inhibit the signaling of IL6, but also to interfere with the function of growth hormone receptors, specifically the IGF1 receptor (Haddad et al., 2005). However, there is some evidence that induction of SOCS3 can help to alleviate symptoms of autoimmune diseases making the functions of IL6 and SOCS3 in disease progression still highly controversial (Ishihara & Hirano, 2002).

2.13 Exercise-Induced Interleukin-6

Contrary to the proinflammatory functions and skeletal muscle wasting of IL6 listed above, there are data to suggest that IL6 may actually be synthesized and secreted by skeletal muscle in response to exercise to promote beneficial effects on metabolism, skeletal muscle hypertrophy, and inflammation (Carson & Baltgalvis., 2010; Lutosławska, 2012). Ostrowski et al. (1998) first theorized that skeletal muscle was the main source of IL6 synthesized and released during the conclusion of the Copenhagen marathon. Before the race, basal levels of IL6 were 1.5 pg/mL ± 0.7 pg/ml and sharply increased to 94.4 pg/ml ± 12.6 pg/ml post-race. An almost 100-fold increase in plasma levels of IL6. Ostrowski et al. (1998) originally thought the drastically elevated levels of plasma IL6 could be due to its secretion by blood mononuclear cells (BMNCs) like monocytes or macrophages, but analysis of these cells found no detectable IL6 mRNA.

Steensberg et al. (2000) furthered this theory when they had human participants perform one-legged concentric leg extensions for 5 hours at 40% of their knee extensor peak power output while femoral arterial and venous catheters were placed to measure the changes in plasma IL6 concentrations. Arterial plasma concentrations of IL6 increased from 0.74 ng/min to 14.14 ng/min after 5 hours of single-leg extensions. There was a gradual increase in plasma concentrations of IL6 until the 3-hour mark followed by an almost exponential increase. Net IL6 release in the exercising leg was significantly higher than the net release in the resting leg. The increase in IL6 plasma concentrations could also be attributed to solely the exercising leg musculature.

Interleukin-6 may be regulated in skeletal muscle via contraction-induced release of calcium and an increase in the levels of reactive oxygen species (ROS) via nuclear factor kappa B (NF-κβ) and heat shock proteins that respond to a variety of factors like oxidative stress, low glucose levels, and increased intracellular calcium levels (Pedersen & Fischer, 2007). This could mean that supplementation with antioxidants would blunt the IL6 response in the exercise-induced model.

IL6 mRNA content in skeletal muscle is lower in chronic exercisers compared to sedentary populations which is theorized to increase the sensitivity of muscle to IL6 signaling and muscle disuse may lead to a resistance of IL6 (Pedersen, 2013). High circulating levels of IL6 are associated with multiple chronic diseases like obesity and resistance to IL6 signaling could be a reason why the body secretes more of it in a potential compensatory mechanism.

2.14 Exercise-Induced Interleukin-6 and Skeletal Muscle Hypertrophy

Exercise-induced IL6 secretion from skeletal muscle has vastly distinct functions related to protein synthesis, metabolism, and inflammation previously listed. Specifically, IL6 has been

shown to promote myogenic determination and differentiation in exercising muscle, promote hypertrophy via activation of satellite cells, and function as an energy sensor responsible for maintaining homeostasis throughout the body (Hoene et al., 2013; Keller et al., 2001; Serrano et al., 2008).

In regard to muscle hypertrophy, it has been shown that IL6 is a potent activator of muscle satellite cells (SCs) which are important for muscle repair following damage and hypertrophy given the correct stimulus. Due to multinucleated myofibers being permanently differentiated and incapable of mitotic division, additional genetic material during post-natal muscle growth relies on the accretion of new nuclei during muscle growth from SCs (Serrano et al., 2008). Normally SCs are dormant and are located between the sarcolemma and basal lamina, but under conditions like muscle damage or resistance training, they become activated, proliferate, migrate, and incorporate themselves into adult myofibers leading to growth and repair (Toth et al., 2011). SCs play an integral role in the process of muscle hypertrophy and hyperplasia.

Serrano et al. (2008) found that after overloading exercise in mice there was an increase into the wild type (WT) mice's cross-sectional area (CSA) in the exercising muscle, but a blunted response in mice who had their IL6 gene knocked out. The WT mice also had a 40% increase in the number of their myonuclei compared to IL6 knockout (KO) mice after 14 days of muscle overloading and maintained this level for 42 days after treatment. They also found a 50% reduction in proliferation rates in IL6 KO mice compared to WT which could be explained by the decreased expression of cyclin-D1, indicating an arrest of the cell cycle (Serrano et al., 2008).

Toth et al. (2011) found that after muscle lengthening contractions (MLC) in humans there was a 200% increase in serum IL6 concentration 24 hours post MLC which much higher than was reported by Ostrowski et al., but this could be due to the eccentric nature of the exercise leading to more muscle damage and an increased need for the activation and proliferation of SCs. SC activation and proliferation was found via staining for the transcription factor Pax7 which is important for SC self-renewal, survivability, and proliferation. Pax7 is also responsible for the transcription of MyoD and Myf5 which are important for cell determination and differentiation (Serrano et al., 2008). Indeed, mice who had their Pax7 genes deleted had muscle weight 1.3-fold lesser than WT, 1.8-fold smaller myofibers, 3.3-fold decreased number of myonuclei, and a 7.5-fold increase in immature myofibers. These mice also have poor survivability and die within 3 weeks of birth (Von Maltzahn et al., 2013).

Interleukin-6 signaling induces an increase in MyoD and Myf5 expression in SCs. After muscle overloading, there was a 1.8-fold increase in Myf5 expression in SCs over a 24-hour period post-MLC. IL6 KO mice have been shown to have decreased expression of MyoD compared to WT (Serrano et al., 2008; Toth et al., 2011). IL6 KO severely reduced the number of SC cells expression MyoD in SCs of muscles undergoing overloading exercise.

As previously discussed, IL6 transduces its signal via its receptor complex and the JAK/STAT3 pathway. IL6 may be implicated in the PI3K/AKT pathway previously associated with myogenesis, however it is widely accepted that signaling through the STAT3 pathway is the primary method in which IL6 activates SCs for myoblast proliferation and gene expression (Serrano et al., 2008). Downstream targets of IL6/JAK/STAT3 signaling include cyclin-D1 and c-Myc, which are known to promote cell growth and proliferation. Serrano et al., (2008) found deletion of the IL6 gene in mice did not produce and reduction of fiber size in adult mice in the

basal state which indicates the mechanisms involved in maintaining fiber size in mature muscles are IL6-independent.

2.15 Interleukin-6 as an Energy Sensor

Interleukin-6 plasma level concentrations in response to exercise has been shown to vary depending on the glycogen content of skeletal muscles prior to exercise (Keller et al., 2001). When muscle is glycogen depleted prior to exercise, there has been shown to be an increase in the slope and magnitude of IL6 secreted into the plasma. Keller et al. (2001) noticed when they had participants undergo dynamic two-legged knee extensor exercise there was a significant increase in the amount of IL6 secreted from the muscle of participants who's muscles were depleted of glycogen compared to the control. Glycogen depleted participants had basal plasma concentrations of IL6 pre-exercise values of 0.7 ng/L ± 0.1 ng/L compared to the control of 0.6 ng/L ± 0.2 ng/L. However, after 120 minutes of exercise the glycogen depleted group had plasma concentrations in IL6 of 8.3 ng/L ± 1.9 ng/L compared to the control which had 3.8 ng/L ± 1.1 ng/L. Additionally, the glycogen depleted group had their IL6 levels remain higher throughout the 180-minute exercise protocol and peaked at 10.3 ng/L ± 1.3 ng/L compared to the control at 6.3 ng/L ± 0.7 ng/L.

The increase in plasma concentration of IL6 from exercising muscle is theorized to work as an energy sensor for skeletal muscle by signaling the liver to increase gluconeogenesis and glycogenolysis to be secreted into the blood stream to be taken up by working skeletal muscle (Gomarasca et al., 2020). *In vitro* and *in vivo* models show that IL6 enhances endogenous glucose production most likely through the AMPK pathway by increasing cAMP and the AMP:ATP ratio but may also work via the PI3K pathway (Li et al., 2017; Pedersen, 2013).

The magnitude of IL6 secretion by working skeletal muscle, however, can be attenuated when those who are exercising consume carbohydrates prior to or during exercise, indicating that glucose and glycogen play a role in the exercise-induced regulation of IL6 secretion (Pedersen, 2013). IL6 also increased glucose uptake by stimulating enhanced insulin secretion via glucagon-like peptide 1 (GLP-1) from intestinal L-cells and pancreatic α-cells (Guo et al., 2017). Acute treatment of muscle with IL6 has been shown to increase basal glucose uptake via translocation of the glucose transporter type 4 (GUT4) from the intracellular compartments of the plasma membrane (Pedersen, 2011).

Interleukin-6 has also been identified as a lipolytic factor. Van Hall et al. (2003) found that recombinant human IL6 (rhIL6) infusion increases fat oxidation and lipolysis. They also noticed that after 2-hours of rhIL6 infusion, energy expenditure in participants could be explained primarily through fat oxidation. They further validated these results due to the increased lipolysis in the absence of hypertriglyceridemia, changes in catecholamines, glucagon, and insulin, with a modest elevation of cortisol levels. These roles of IL6 as an energy sensor help to establish it as an important exercise factor.

2.16 Interleukin-6 as an Anti-Inflammatory Myokine

With IL6's role as a proinflammatory cytokine discussed extensively, its recent classification and functions as a myokine have shed light on the anti-inflammatory effects of IL6 in response to exercise. Interleukin-6 has been shown to regulate inflammation but is not the primary cause. Interleukin-6 is classified as an inflammatory responsive cytokine for its multiple roles promoting and terminating factors causing inflammation (Ostrowski et al., 1999). Interleukin-6 secretion during infection and exercise have different effects on inflammation and the factors secreted from different tissues involved in communication throughout the body.

During exercise, unlike damage or infection, there is no rise in tumor necrosis factor α (TNFα) preceding elevation in IL6 plasma concentrations (Ostrowski et al., 1999). Rather, during exercise there is an elevation in the concentration of anti-inflammatory cytokines and signals that inhibit proinflammatory cytokines like TNFα and interleukin-1β through secretion of interleukin-1 receptor antagonist (IL-1RA) and interleukin-10 (Ostrowski et al., 1999). C-reactive protein (CRP), as briefly described above in the diagnosis of SCI is only moderately detected after marathon running unlike during infections that increase its levels significantly.

The anti-inflammatory roles of IL6 seem to function when it interacts with cells that express the mIL6R receptor rather than the trans-signaling functions accompanied with the sIL6 receptor (Lightfoot & Cooper, 2016). The inflammatory effects of IL6 also seem to change depending on the concentration of TNFα present during inflammatory responses to stimuli (Gomarasca et al., 2020). This coincides with exercise not elevating the plasma concentration of TNFα compared to other inflammatory stimuli briefly described above. There is also some evidence that exercise induced IL6 can attenuate almost all of the side effects associated with the proinflammatory IL6 (Daou, 2020).

2.17 Conclusion

The pleiotropic and sometimes controversial effects of IL6 make it an important molecule to understand for the prevention of different disease pathologies as well as understanding more beneficial effects of exercise. Although evidence suggests skeletal muscle is able to secrete IL6, there are still questions as to whether it is synthesized in skeletal muscle during exercise and which mechanism is driving this synthesis and consequent secretion into the blood stream.

Pedersen (2011, 2013) theorized that IL6 is expressed by muscle cells. An *ex vivo* protocol should be utilized to evaluate this theory which will eliminate circulating factors like

immune cells which could be contributing to the expression of IL6 during exercise and binding of circulating IL6 to the working skeletal muscle.

CHAPTER III

METHODS

3.1 Animals and Treatment

The primary focus of this study was to determine if there is a significant increase in skeletal muscle expression of IL6 in response to exercise using an *ex vivo* muscle contraction model. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Northern Colorado (UNC) and were conducted in accordance with the Animal Welfare Act. Six male Sprague-Dawley rats were obtained from the UNC breeder colony and euthanized using heparinized (100U) sodium pentobarbital (50 mg/kg). When a tail pinch was absent, the right and left soleus (SOL) and extensor digitorum longus (EDL) muscles were excised. The right sided SOL and EDL muscles were used for the exercise condition. The left sided SOL and EDL for each rat functioned as the control (or sedentary) group and was flash frozen immediately in liquid nitrogen and stored at -80°C for biochemical analysis. After removal of the SOL and EDL muscles, the heart was excised to ensure death.

3.2 Treatment of Right Soleus and Extensor Digitorum Longus Muscles

Skeletal muscle contraction was accomplished *ex vivo* using an Radnotti organ bath system outfitted for skeletal muscle contraction assessment (AdInstruments, Colorado Springs, CO). After muscles were excised, they were placed in warm (37°C), oxygenated (95% O_2/5% CO_2) Krebs-Henseleit buffer (120 mM NaCl, 5.9 mM KCL, 2.5 mM $CaCl_2$, 1.2 mM MgCl, 25 mM $NaHCO_3$, 17 mM glucose) and allowed to stabilize. After stabilization, sutures with micro-spring clips were attached to the distal and proximal tendons of each muscle. The proximal end

of the muscle was attached to an isometric force transducer and the distal end of the muscle was attached to a stationary glass hook. Muscles were submerged at resting length in an organ chamber filled with 37°C Krebs-Henseleit buffer with stimulating electrodes mounted on the proximal and distal sides of the muscles.

Following stabilization, optimal length and optimal stimulation voltage were determined by analyzing isometric twitch contraction force at progressively increasing lengths. An initial tension of 0.5 g (measured via force transducer) was applied to the muscle and contraction was stimulated using field stimulation at 40 V. Isometric twitch force was recorded using a Power Lab data acquisition system (ADInstruments). Muscle tension was increased by 0.2 g per trial until an increased tension did not elicit an increase in twitch force.

After maximal twitch force determination, the Krebs-Henseleit buffer was refreshed, and the right SOL and EDL muscles were allowed a 30-minute recovery period before the fatiguing protocol was administered. Fatigue rate was determined at optimal tension. Stimulation was provided at 40 V with a frequency of 83 Hz and a pulse duration of 500 ms. Muscles were stimulated to contract every second for 100 seconds and forces throughout the protocol were recorded. Following the fatiguing protocol, samples were flash frozen in liquid nitrogen and stored at -80°C for ensuing biochemical analysis.

3.3 Biochemical Analysis

Analysis of each sample was done utilizing an enzyme-linked immunosorbent assay (ELISA; R&D Systems Inc; Minneapolis, MN) to quantify the expression of IL6. Soleus and EDL samples were removed singularly from the -80°C freezer and ~0.5 g of tissue was sectioned from each muscle sample. For SOL muscles, a 1:10 ratio (weight to volume) of radio-immunoprecipitation (RIPA) lysis (Santa Cruz Biotechnology: Santa Cruz, CA) was added,

along with 10µL of protease enzyme inhibitor (Sigma-Aldrich). For EDL muscles a 1:5 ratio (weight to volume) of RIPA was added along with 10µL of protease enzyme inhibitor. All samples were then manually homogenized in a handheld glass tissue homogenizer. Tissue samples were centrifuged for 10 minutes at 10,000 rpm at room temperature. After centrifugation, the supernatant was collected for analysis and the pellet was discarded.

3.4 Bradford Assay

The Bradford method (Bradford; Coomassie Plus Protein Assay Reagent, ThermoScientific; Rockford, IL) was utilized to quantify the total protein concentration in the supernatant. Known concentrations of BSA (pre-diluted protein assay standards; Bovine serum albumin, ThermoScientific: Rockford, IL; 2 mg/ml, 1 mg/ml, 0.5 mg.ml, 0.25 mg/ml, and 0.125 mg/ml) were used with spectrophotometry to create a standard curve for protein concentration and absorbency. 1000 µL of Bradford reagent was added to each sample. The spectrophotometer was set at a wavelength of 595 nm, and the standard curve was established. Next, 10 µL of each sample was loaded in the spectrophotometer. Protein concentration of each sample was predicted using the linear equation $x=y-b/m$ based on the standard curve. When protein concentrations were calculated for each sample, 100 µL of supernatant was added to a calculated amount of RIPA needed for each sample. This was based on calculated volume of protein content for 2 mg/mL from a 100µL aliquot.

3.5 Enzyme-Linked Immunoassay

Enzyme-linked immunoassay (ELISA) was utilized for IL6 measurements (R&D Systems co. Minneapolis, Minnesota). All reagents and samples were brought to room temperature prior to use. All standards, control, and samples were assayed in duplicate. 50 µL of Assay Dilutant RD1-54 was added to each well. 50 µL of standard or sample was added to each

well in duplicate. Wells were mixed by gently tapping on the plate frame for 1 minute. Wells were then covered with an adhesive strip and incubated for 2 hours at room temperature. After incubation, each well was aspirated and washed five times. Wells were washed by filling each well with wash buffer using a squirt bottle with complete removal of liquid at each step. After the last wash, any remaining wash buffer was removed by aspiration. The wells were then inverted and blotted against a clean paper towel.

100 µL of Rat IL6 conjugate was added to each well. Wells were covered with a new adhesive strip and incubated at room temperature for 2 hours. After incubation, aspiration and washing steps were duplicated as discussed previously. 100 µL of substrate solution was added to each well. Wells were incubated in the dark for 30 minutes. 100 µL of stop solution was then added to each well. Wells were tapped gently to ensure thorough mixing.

Samples were analyzed within 30 minutes of stop solution being added using a microplate reader. Dual wavelength corrections were set to 540 nm to 570 nm to properly illuminate protein content in each well. Protein concentrations were predicted using the linear equation $y=mx + b$ based on the standard curve previously established using standard samples.

3.6 Statistical Analysis

Data are present as means ± SEM. An unpaired t-test was utilized to determine differences between sedentary and exercising muscles. Significance was established at $p < 0.05$.

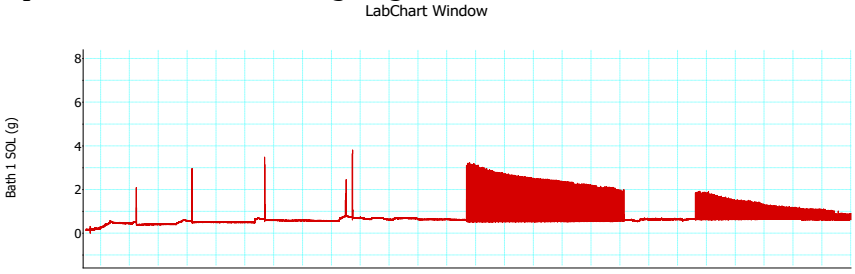
CHAPTER IV

RESULTS

4.1 Optimal Tension Protocol

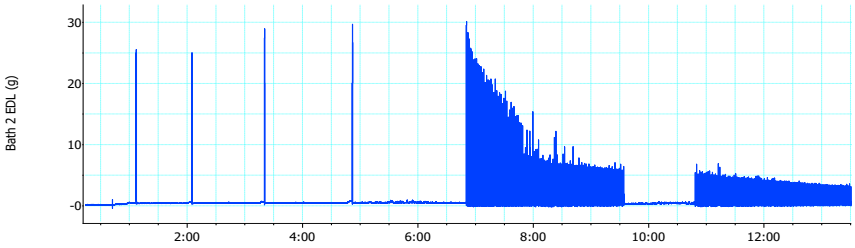
Maximal twitch contraction force for SOL can be seen in Figure 1. Extensor digitorum longus muscles can be observed in Figure 2. As previously stated, muscle peak contraction force was measured at progressively longer lengths until a peak twitch force was determined. An initial tension of 0.5 g was utilized, and muscles were lengthened by 0.2 g until increased force production was not observed. Extensor digitorum longus muscles produced a much higher peak contraction force than SOL muscles. However, SOL muscles were shown to be more resistant to fatigue as their peak force was maintained longer than the EDL muscles.

Figure 1.
Optimal Tension and Fatiguing Protocol SOL Muscles



Note. SOL muscles produced less force than EDL muscles (Figure 2), but were more resistant to fatigue

Figure 2
Optimal Tension and Fatiguing Protocol EDL Muscles



Note. EDL muscles produced a higher peak force than SOL muscles (Figure 1) but fatigued much quicker.

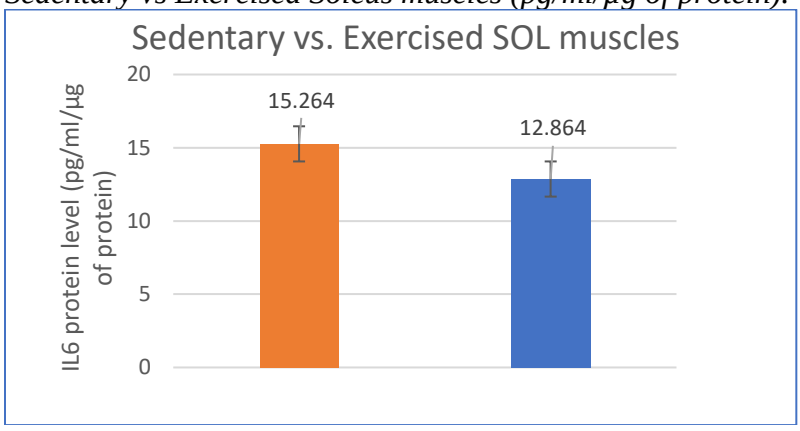
4.2 Fatiguing Protocol

Results of the fatiguing protocol for each muscle can be seen in Figures 1 and 2. Muscles were stimulated to contract at their maximal twitch contraction every second for 100 seconds and muscle fatigue was observed. Extensor digitorum longus muscles experienced more of a relative decline in peak twitch force compared to SOL muscles most likely due to fiber type differences between muscles. Soleus muscles are primarily characterized by a higher proportion of type I muscle fibers compared to EDL muscles which predominantly contain higher concentrations of type II muscle fibers. However, EDL muscles had higher force production compared to SOL muscles in the initial stages of the protocol.

4.3 Biochemical Analysis

Data are presented as means ± SE. There was no significant difference in protein levels between exercised EDL muscles and sedentary EDL muscles (Figure 3) ($p = 0.41$). There was no significant difference between sedentary SOL muscles compared to exercised SOL muscles (Figure 4) ($p = 0.328$). Although not significant, there does appear to be a trend towards exercised muscle (SOL and EDL) having less protein content of IL6.

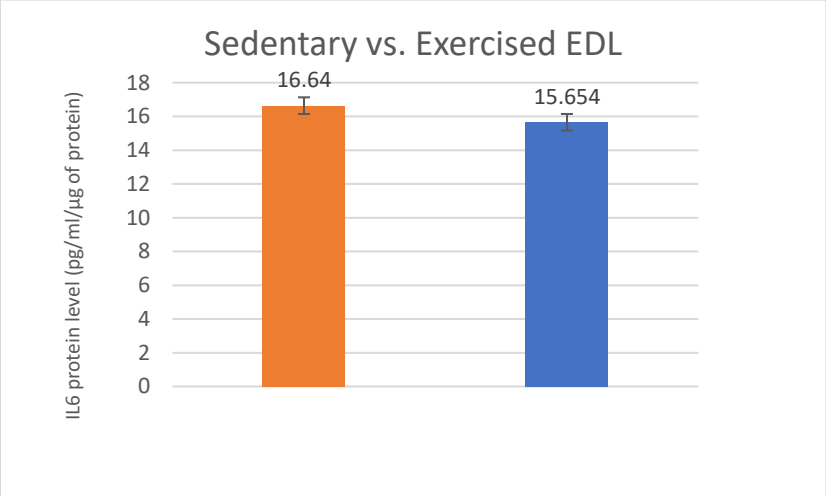
Figure 3.
Sedentary vs Exercised Soleus muscles (pg/ml/µg of protein).



Note. There was no significant difference between the two groups ($p = 0.328$)

Figure 4

Sedentary vs Exercised Extensor Digitorum Longus Muscles



Note. There were no significant differences between sedentary and exercised muscle groups (p = 0.41).

CHAPTER V

DISCUSSION

5.1 Summary

Interleukin-6 is a pleiotropic cytokine with roles in regulating the immune system, glucose metabolism, and muscle physiology (Keller et al., 2001; Serrano et al., 2008). Previous research has shown a robust increase in IL6 secretion in the blood stream following exercise and skeletal muscle itself may be responsible for these elevated levels (Ostrowski et al., 1998; Steensberg et al., 2000). Secretion of IL6 by skeletal muscle in response to exercise has been shown to produce anti-inflammatory functions contrary to its proinflammatory effects normally seen (Pedersen, 2011; Xing et al., 1998). Interleukin-6 when secreted by skeletal muscle in response to exercise has been shown to increase glucose uptake by the working skeletal muscle, increase muscle hypertrophy, and decrease systemic inflammation (Pedersen, 2012; Serrano et al., 2008; Van Hall et al., 2003).

Understanding the functions of IL6, specifically its mechanism of action in response to exercise, is important for determining ways mitigating the rise of non-communicable chronic diseases. Specifically, chronic inflammatory diseases have been recognized as the most significant causes of death in the world in recent years (Furman et al., 2019). Conditions like ischemic heart disease, stroke, cancer, type II diabetes (T2D), kidney disease, autoimmune diseases, and neurodegenerative diseases are on the rise globally with many having root causes in physical inactivity and a sedentary lifestyle (Furman et al., 2019; Lavie et al., 2019).

The purpose of this study was to examine if skeletal muscle expression of IL6 changes in response to exercise *ex vivo*. Our study found there was no significant change in the amount of IL6 being expressed in exercised skeletal muscle when compared to sedentary muscle. Although not significant, there appeared to be a trend toward exercised muscle having decreased levels of IL6 present in the skeletal muscle. This phenomenon could be explained by increased signaling in the skeletal muscle cell to release IL6 into the bloodstream. Therefore, exercise could actually be causing less available IL6 in skeletal muscle. Our experiment was also limited to measuring for muscle protein IL6, whereas IL6 mRNA levels were not measured. Future experiments should be conducted to determine whether protein IL6 concentration and mRNA concentration follow similar patterns.

5.2 Potential Mechanisms of Interleukin-6 Expression and Secretion

Interleukin-6 appeared to be consistently expressed in both muscle groups which supports literature that skeletal muscle does express IL6. Although, there was no significant difference between groups, this finding could signify that IL6 is constantly expressed in skeletal muscle, but skeletal muscle contraction was not a mechanism to significantly increase expression of IL6. Pedersen and Fischer (2007) speculate there may be a few mechanisms by which IL6 levels are increased during exercise with calcium-induced muscle contraction being one. They speculate things like nuclear factor kappa beta (NFκβ), a redox sensitive transcription factor; low glycogen environments, and reactive oxygen species (ROS) may be other mechanisms that can enhance the expression of IL6 in response to exercise.

Muscle glycogen levels, reactive oxygen species (ROS) levels, and the activity of nuclear factor kappa beta (NFκβ) may also signal an increase in IL6 expression and secretion in response to exercise (Keller et al., 2001; Pedersen & Fischer, 2007). Glycogen content in skeletal muscle

has been shown to be a major factor in determining whether there will be a significant elevation in IL6 expression and secretion into the blood stream (Keller et al., 2001; Pedersen, 2012). When there are low levels of glycogen in skeletal muscle there is a significant elevation in IL6 secreted into the bloodstream. This is theorized to communicate with the liver to release more glucose for working muscle and increase the rate of glucose uptake by skeletal muscle via AMPK and GLUT4 (Pedersen, 2012). Prior research has also found that when participants are fed a high glucose meal prior to exercise there is not as high of an IL6 secretion into the bloodstream compared to those who did not consume glucose prior to exercise (Keller et al., 2001). In fact, IL6 secretion by the low carbohydrate group actually blunted the IL6 accumulation in the bloodstream.

Reactive oxygen species are thought to be another mechanism that could influence the expression and secretion of IL6 from skeletal muscle. Intense exercise is known to cause an increase in ROS levels mostly from the mitochondria, electron transport chain (ETC), NADH-oxidase and xanthine oxidase (Kosmidou et al., 2002). Kosmidou et al., (2002) established that skeletal muscle exposed to stress from ROS similar to levels seen at intense exercise elicited an increase in IL6. They also demonstrated that myotubes exposed to ROS experienced a 12.2-fold increase in IL6 expression and there seemed to be a concentration -dependent mechanism that also depends on the concentration of antioxidants present in the muscle cell. Exogenous H_2O_2 when added to muscle cells activates a cascade of serine/threonine kinases that work to inhibit tyrosine phosphatases. These events lead to growth factors being expressed in the cell, and this is thought to regulate the amount of IL6 expressed in the cell (Kosmidou et al., 2002).

The buildup of ROS from exercise is thought to increase the activity of the transcription factor NF-κβ (Kosmidou et al., 2002). Nuclear Factor Kappa Beta performs a crucial step in

activating inflammatory genes and the production of IL6 (Brasier, 2010). Libermann and Baltimore (1990) found the IL6 promotor includes an NF-κβ binding site. They also demonstrated that NF-κβ can be activated by a wide array of inflammatory agents and ROS contributing to an increased expression of IL6 regulation. They also speculate that the activation of NF-κβ may represent a key step in the regulation of IL6 expression.

However, it has also been shown by Liu and Chang (2018) that moderate exercise actually causes a suppression of NF-κβ in mice with T2D. The suppression of NF-κβ with moderate exercise then corresponds to a decrease in IL6 blood concentration (Liu & Chang, 2018). While increased levels of NF-κβ are seen in high-intensity exercise, this is seen as contributing to muscle protein degradation via the buildup of pro-inflammatory cytokines like IL6. Therefore, looking to NF-κβ as a regulator in the positive effects of IL6 during exercise may not be optimal. However, the mechanism with which there is elevated IL6 expression and secretion merits future research.

5.3 Future Research

Future research in studying IL6 expression and secretion via skeletal muscle expression and secretion should continue to be studied and monitored. Specifically, how several types of exercise influence IL6 expression and secretion into the bloodstream and how these effects could be beneficial to groups suffering from muscle wasting disorders. Future research should try to determine which specific mechanism(s) are needed for IL6 expression to increase and if it is being secreted into the bloodstream by skeletal muscle.

Research should also focus on whether there is a difference between resistance trained and aerobic trained IL6 expression and secretion after exercise and which intensity is needed. Our study analyzed the effects of a fatiguing muscle protocol on IL6 expression fibers; however,

future research should aim to observe the effects of resistance training versus aerobic training on IL6 response to exercise.

Ostrowski et al. (1998) have already established that marathon running provides sufficient intensity to garner an elevated level of IL6 in the bloodstream. However, this mode of exercise is not always practical from a general population standpoint due to time constraints and the amount of training needed for training. There is evidence that Olympic Weightlifting increases the amount of IL6 present in the bloodstream from moderate and intense resistance training (Kaniganti & Majumdar, 2019). More research on resistance training intensity, type, and length should be studied due to its more practical approach. However, careful coaching and supervision may be needed to ensure proper form and loading in order to avoid injury.

5.4 Conclusion

In this study muscles excised from rats and stimulated to contract at peak force until fatigued did not result in a significant IL6 difference in in exercised versus control rats ($p > 0.05$). While a trend was noticed that exercised muscles had less IL6 protein content, this could be due to secretion of IL6 as a result of muscle contraction stimulus.

Exercise has multiple factors and mechanisms to improve muscle and metabolic adaptations. While IL6 continues to be studied, it should still be recommended that individuals exercise for the purpose of health benefits and to stave off preventable non-communicable diseases.

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APPENDIX A

INSTITUTIONAL ANIMAL CARE & USE COMMITTEE APPROVAL

UNIVERSITY OF NORTHERN COLORADO

IACUC Memorandum

To: David Hydock
From: Laura Martin, Director of Compliance and Operations
CC: IACUC Files
Date: December 28, 2017
Re: IACUC Protocol Approval, 1711CE-DH-R-20

The UNC IACUC has completed a final review of your protocol "Nutrition and Exercise in Cancer Treatment-Induced Muscle Dysfunction".

The protocol review was based on the requirements of Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training; the Public Health Policy on Humane Care and Use of Laboratory Animals; and the USDA Animal Welfare Act and Regulations. Based on the review, the IACUC has determined that all review criteria have been adequately addressed. The PI/PD is approved to perform the experiments or procedures as described in the identified protocol as submitted to the Committee. This protocol has been assigned the following number 1711CE-DH-R-20.

The next annual review will be due before December 28, 2018.

Sincerely,

Laura Martin, Director of Compliance and Operations