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The Inflammatory Response to Acute Muscle Injury

Kevin O'Fallon

University of Massachusetts - Amherst

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THE INFLAMMATORY RESPONSE TO ACUTE MUSCLE INJURY

A Dissertation Presented

by

KEVIN S. O’FALLON

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

February 2014

Kinesiology
THE INFLAMMATORY RESPONSE TO ACUTE MUSCLE INJURY

A Dissertation Presented

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KEVIN S. O’FALLON

Approved as to style and content by:

Lawrence M. Schwartz, Chair

Edward Debold, Member

Young-Cheul Kim, Outside Member

Monica Hubal, Outside Member

Patty Freedson, Department Head
Kinesiology
DEDICATION

This dissertation is dedicated to the memory of my Mentor, Dr. Priscilla M. Clarkson who saw in me what I have only begun to see in myself, through hard work and perseverance. It is an honor and a privilege to have been her student and to have known her. Dr. Clarkson taught me more about myself than I would have learned in a lifetime on my own. I am ever grateful for her unwavering commitment and tremendous contributions to my professional development and personal growth.
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I owe a million thanks to my wife Tracy for enduring this with me. You were truly in the trenches with me every step of the way. Your love, patience, and understanding throughout this process have been incredible. I am eternally grateful for you and for the sacrifices you have made in support of this endeavor.

To all of my friends and extended family, I thank you for always believing in me and for your constant support. To my mentors and colleagues, you have all inspired me to do my best every day, sometimes, just for the sake of trying to keep up with you. I thank you.

Thank you to my committee members especially Drs. Larry Schwartz and Monica Hubal, who have made significant contributions to my work and to my development into a scientist. I would also like to acknowledge the Department of Defense, the United States Army, for this and the Natick Soldier RDEC Combat Feeding Directorate for funding this Dissertation.
ABSTRACT

THE INFLAMMATORY RESPONSE TO ACUTE MUSCLE INJURY

FEBRUARY 2014

KEVIN S. O’FALLON, B.S., UNIVERSITY OF MASSACHUSETTS AMHERST

M. S., UNIVERSITY OF MASSACHUSETTS AMHERST

Ph.D., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor Lawrence M. Schwartz

The overall goal of this dissertation was to examine inflammatory and regenerative responses to acute skeletal muscle damage and to define molecular mediators of repair. Study I examined the effects of an oral anti-inflammatory supplement on exercise-induced muscle damage (EIMD) and systemic inflammation in a human model. Quercetin has been shown in animal and in vitro models to downregulate nuclear factor-kappa beta (NF-κB) nuclear transactivation and monocyte chemoattractant protein 1 (MCP-1) secretion, which regulate muscle regeneration and inflammatory signaling between muscle and immune cells after injury. Subjects ingested quercetin (N=15) or placebo (N=15) before and after performing 24 eccentric contractions of the elbow flexors. Subjects experienced moderate strength losses and delayed onset muscle soreness, indicating damage, but no supplementation effect was observed. The null effect of quercetin in the human model (with its complex inflammatory response) encouraged us to explore basic injury-induced inflammation in a controlled in vitro model, to better understand the post-injury roles of NF-κB and MCP-1.
Study II used an *in vitro* injury model (scratch of C₂C₁₂ myotubes) to identify the roles and interplay of NF-κB and MCP-1 in muscle regeneration and inflammation following acute injury.

Protein expression changes of NF-κB and MCP-1, and morphological changes in regenerating muscle cultures were monitored for 24-72 hours (h) post-injury (3-6 replicates per experiment). NF-κB activation was significantly downregulated (-30±1.4% to -44±1.1%) at 6-12h post-injury. Pharmacological blockade of NF-κB downregulated satellite cell proliferation by 19±9% after 19h and 72h, evidence for a role of NF-κB signaling in post-injury regeneration. Furthermore, NF-κB activation strongly correlated (R=0.69) with MCP-1 secretion from injured muscle cultures, and blockade of NF-κB reduced MCP-1 secretion at 1–24h (-33±0.1%) and strongly correlated (R=0.74) with NF-κB activation. These data support recent *in vivo* findings to demonstrate that NF-κB and MCP-1 signaling are critical regulators of inflammatory and regenerative responses following muscle injury. Moreover, this work provides the first kinetic profile of early (<24 hours) molecular responses of NF-κB and MCP-1 to acute muscle injury, and introduces novel evidence that NF-κB regulates MCP-1 protein secretion, indicating an indispensible role of NF-κB signaling in muscle inflammation *in vitro*. 
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CHAPTER I
INTRODUCTION

The overall goal of this dissertation was to examine inflammatory and regenerative responses to acute skeletal muscle damage and to define molecular mediators of repair. Eccentrically-biased exercise is well known to cause ultrastructural damage to skeletal muscle fibers and impair muscle function (24, 26, 44, 59). Exercise-induced muscle damage (EIMD) involves the complex processes of initial muscle degeneration and secondary (likely inflammation-induced) damage, and is typically followed by a period of regeneration, which involves activation of satellite cells and repair of damaged myofibers (75, 80, 82, 83). Inflammation plays important roles in both the degenerative and regenerative responses to EIMD (48), specifically in the communication between the injured tissue and the immune cells that are recruited to facilitate debris removal (degeneration) (45, 46, 105), activation of satellite cells (9, 89), and subsequent repair of injured myofibers (90, 103, 108). Although some evidence from human EIMD models suggests that targeting prevention or attenuation of inflammation with anti-inflammatory agents can accelerate muscle repair (17, 104), little is actually known about the underlying mechanisms of inflammation and the net contribution of inflammation to muscle regeneration after strenuous exercise and injury.

The goal of my first study was to determine the effects of a plant-based anti-inflammatory compound (quercetin) on systemic indirect markers of EIMD and inflammation following 24 eccentric actions of the elbow flexors in a human model. The exercise bout induced a moderate level of muscle damage, measured via strength loss and delayed onset muscle soreness. Although oral quercetin supplementation did not
attenuate typical changes in markers of EIMD and inflammation, these results encouraged me to focus further studies on the molecular pathways that quercetin was purported to target, to determine underlying mechanisms of initial muscle inflammation and regeneration at the cellular and molecular levels.

Evidence from previous studies (58, 71, 73) suggested that quercetin, through its inhibition of NF-κB/p65 activation (58, 71) and modulation of MCP-1 gene and protein expression (73), inhibits the pro-inflammatory/degenerative activity of monocytes and macrophages, and in turn, may reduce secondary damage caused by inflammation. While quercetin may or may not significantly modify these processes in EIMD, there is substantial evidence that NF-κB activation and MCP-1 expression play important roles in the initial signaling events between muscle cells and monocytes and macrophages following muscle injury, although mechanisms driving these processes (and how they related to each other) are poorly understood. Therefore, the goal of my second study was to explore the specific roles of NF-κB activation and MCP-1 expression within skeletal muscle cells following acute injury in a simple model system. Using an isolated in vitro scratch-injury model of cultured C_{2}C_{12} myotubes (containing both satellite cells and myotubes), I explored: 1) NF-κB activation and MCP-1 protein expression and secretion from injured myotube cultures to determine their specific roles; and 2) the interplay between NF-κB activation and MCP-1 expression in the inflammatory and regenerative responses to injury.

The inflammatory and regenerative responses to muscle injury have been widely studied at the systemic and local muscle level in human, animal, and in vitro models. However, the systemic inflammatory response to damage is often not representative of
the early molecular signaling events that occur within the injured tissue. In this
dissertation, I first used a human EIMD model to explore changes in the expression of
typical damage and inflammatory markers following EIMD and how they might be
affected by anti-inflammatory supplementation. The data from Study I brought me to the
realization that taking the systemic approach to understanding EIMD in vivo wouldn’t
reveal specific information about how the muscle cells themselves responded to damage,
so, I returned to the literature and identified two molecular processes that would be more
representative of the intrinsic muscle response to damage: NF-κB activation and MCP-1
expression. In Study II, I examined those processes in a simplified in vitro model using
cultured C2C12 muscle cells.

Study I

Eccentric muscle contractions during bouts of strenuous exercise are well known
to produce decrements in muscle function, such as delayed onset muscle soreness and
prolonged losses in strength and range of motion in the days following the exercise (12,
37, 66). These manifestations are often associated with inflammation and increased
oxidative stress, likely from reactive oxygen and nitrogen species (RONS) production
(29). Quercetin is an abundant flavonol-type flavonoid, commonly found in vegetables
and fruits (56). It is purported to act as: 1) an antioxidant to control the harmful effects of
RONS; and 2) an antihistamine to help reduce inflammation. Previous studies have
demonstrated quercetin supplementation (in concert with other bioactive ingredients)
reduced systemic inflammation after damaging eccentric exercise (79). Moreover,
quercetin was also purported to affect the expression of inflammatory proteins from
immune cells that play important roles in muscle regeneration (58, 71, 73) Therefore, the purpose of Study I was to determine if quercetin supplementation would attenuate secondary muscle damage, as evidenced by reduced muscle soreness, swelling, and inflammatory markers in the blood, lower losses of strength and range of motion, and improvements in the overall profile of recovery after strenuous exercise in healthy young men and women. To determine the effects quercetin supplementation on markers of EIMD and systemic inflammation, we utilized an eccentrically-biased exercise protocol that consisted of 24 maximal eccentric actions of the elbow flexors, a model proven to induce moderate muscle damage (53, 54, 67, 68).

The results of Study I indicated that this exercise bout induced a moderate level of muscle damage and typical changes in our markers of EIMD and inflammation such as strength-loss, soreness, and muscle swelling. However, we observed no significant change in these parameters in quercetin treated subjects, suggesting that quercetin supplementation does not affect damage or accelerate recovery from EIMD in humans. Nevertheless, in a parallel series of experiments, I sought to determine how the injured striated skeletal muscle tissue recruits the systemic factors that cause local muscle inflammation and facilitate regeneration after injury.

**Study II**

The limited understanding of the molecular mechanisms that regulate communication between injured muscle tissue and peripheral immune cells (i.e. monocytes and macrophages) during the course of muscle degeneration and regeneration has been cited as a major gap in the scientific literature that needs to be addressed in
order to develop better therapeutics to treat muscle injury and enhance repair (8, 102). Macrophages appear to play a dual role in muscle injury. First, by initially promoting inflammation and secondary muscle damage during the acute inflammatory phase, and then subsequently, by promoting myogenesis and wound healing during the chronic phase of inflammation (103). Furthermore, since MCP-1 is known to induce the migration of monocytes/macrophages into injured muscle (3), and NF-κB activation regulates MCP-1 expression in macrophages (73) and satellite cell differentiation in muscle (5), it is possible that NF-κB and MCP-1 play pivotal roles in regulating muscle inflammation and regeneration after injury (Figure 1.1). Therefore, the overall purpose of Study II was to determine the roles of NF-κB activation and MCP-1 expression and secretion from muscle cells, in regulating the inflammatory and regenerative responses to acute muscle injury.
Figure 1.1. Study II- conceptual model of the roles of NF-κB and MCP-1 signaling in muscle cells, monocytes, and macrophages following acute injury. The solid lines denote what is known from the literature. The black dashed lines (---) indicate apparent gaps in the current knowledge and the green dashed lines (----) indicate new insights from Study II that address gaps in the literature. (1) Muscle damage induces NF-κB transactivation, presumably leading to transcription and translation of cytosolic MCP-1 protein. (2) Secretion of MCP-1 from injured myotubes and satellite cells induces the recruitment of circulating monocytes and activation of pro-inflammatory M1 macrophages. (3) The fate of monocyte differentiation into M1 or M2 macrophages is currently unknown. (4) M1 macrophages promote nitrous oxide (NO)-induced secondary muscle damage and satellite cell proliferation. (5) Anti-inflammatory M2 macrophages reduce M1 macrophage induced muscle cell lysis and promote differentiation and fusion of satellite cells with injured myotubes, which are critical for wound healing and resolution of muscle injury.
Satellite cells can fuse to form synctial muscle fibers. However, skeletal muscle also contains a variety of mononucleated cells that do not contribute directly to muscle fiber formation, such as endothelial cells, fibroblasts, and macrophages. The cell type(s) that respond to muscle injury with altered NF-κB activation and MCP-1 expression are currently unknown, largely due to the complexity of studying interdependent molecular processes in higher order systems such as animals and humans.

Simplified in vitro models serve as useful tools for exploration of complex biological processes, and they typically offer a higher degree of experimental control over in vivo systems. To determine if NF-κB activation and MCP-1 expression originates from injured muscle cells (both myotubes and satellite cells), I adapted two existing in vitro models of muscle injury in isolated (i.e. not containing macrophages) cultures of C2C12 muscle cells for Study II (78, 115). Subsequently, I examined NF-κB/p65 nuclear transactivation and both intracellular MCP-1 protein expression and secretion from myotube cultures during the first 24h post-injury.

The approach I took was to mechanically induce muscle injury by scraping cells from the dishes with a sterile gel-loading pipette tip (see above). I then measured NF-κB activation and MCP-1 expression in muscle cells and the appearance of MCP-1 protein in the culture media at baseline (BSLN), 1, 3, 6, 12, and 24h later. Part of the motivation for these experiments was based on prior evidence that implicated NF-κB activation and MCP-1 expression as important processes in muscle repair following injury (36, 38, 98, 107). As examples, NF-κB activation can directly promote MCP-1 expression in vascular smooth muscle cells in vitro (98), and in damaged human muscle tissue after EIMD (107). Mechanical strain-injury induces MCP-1 secretion from cultured human primary
muscle cells (myotubes) within 3.5h, and *in vivo*, MCP-1 mRNA expression is elevated at 3h (38) and ~6h (36) in human muscle tissue following EIMD. However, there are currently no published data that establish if the elevation in NF-κB activation and MCP-1 secretion following injury originates in skeletal muscle cells themselves or comes from resident cells and/or peripheral immune cell populations that migrate to the injured tissue. Thus, in this dissertation I took advantage of an isolated C2C12 muscle cell model and targeted the first 24h post-injury, a time period when NF-κB activation and MCP-1 expression have been previously associated with inflammation-induced muscle degenerative processes (36, 38, 77, 80, 83, 85, 107, 111).

In Study II, I was able to document *in vitro* the early (<24h) kinetic changes in, and interplay between, NF-κB activation and MCP-1 expression in myotube cultures during the first 24h after acute injury. Moreover, changes in NF-κB activation correlated well with changes in MCP-1 secretion from injured myotube cultures demonstrating a strong relationship between both processes. I also observed that pharmacological inhibition of NF-κB activation decreased satellite cell proliferation, which supports the hypothesis that NF-κB transactivation plays a critical role in muscle regeneration. Taken together, these results suggest a potential role for NF-κB activation in both regeneration and MCP-1-mediated inflammatory signaling from muscle cells.

These data support recent *in vivo* findings demonstrating that NF-κB activation and MCP-1 signaling are critical regulators of inflammatory and regenerative responses in muscle cells to acute injury. Moreover, this work provides the first kinetic profile of early (<24h) molecular responses of NF-κB and MCP-1 to acute muscle injury, and
introduces novel evidence that NF-κB regulates MCP-1 protein secretion, indicating an indispensable role of NF-κB in muscle inflammation in vitro.

**Summary**

The overall goal of this dissertation was to examine inflammatory and regenerative responses to acute skeletal muscle damage and to define molecular mediators of repair. Study I tested the effects of a novel anti-inflammatory compound (quercetin) on systemic markers of EIMD and inflammation following 24 eccentric actions of the elbow flexors in a human model, finding no overt effects of quercetin supplementation at the systemic level. Given the complexity of the inflammation response in humans, I then switched to a simpler model system to test two basic mechanisms thought to modify muscle injury and regeneration, namely NF-κB activation and increased MCP-1 secretion (and the influence of NF-κB activation on MCP-1 secretion).

Study II generated novel in vitro data on the early time course of NF-κB activation and MCP-1 expression following scratch injury in C2C12 cells, finding moderate to high correlations between the two responses, and pharmacological blockade of NF-κB affecting MCP-1 levels and muscle regeneration. Knowing the muscle cell-derived kinetic profiles of NF-κB activation and MCP-1 expression may lead to the development of therapeutic interventions designed to both reduce secondary damage caused by inflammation and to accelerate recovery from muscle injury. My demonstration that NF-κB activation regulates both satellite cell proliferation and MCP-1 expression after injury, provides evidence regarding the dual-role of NF-κB activation in
both muscle regeneration and MCP-1-mediated inflammatory signaling from skeletal muscle cells. Taken together, the findings from this dissertation advance our understanding about two important molecular responses of skeletal muscle cells that regulate regeneration and inflammation following acute injury.
CHAPTER II

REVIEW OF LITERATURE

Introduction

Skeletal muscle is the largest tissue in the body by mass and serves as its primary reservoir of amino acids. The highly structured organization of the contractile apparatus allows muscle tissue to shorten and lengthen under mechanical load and thereby perform work that is the basis of organismal locomotion. Muscle also serves as a biological suit of armor that protects the vital organs and skeletal system from blunt trauma and various forms of injurious insults.

Muscle tissue is not impervious to injury, and external forces in excess of its capacity to withstand or dissipate those forces leads to tissue damage. There are a variety of ways that muscle can be injured and each type of injury results in a corresponding inflammatory response and regenerative program. Moreover, the severity of muscle injury and the ensuing molecular responses occur along a continuum that ranges from least severe to traumatic injury. Mild injuries, such as the micro trauma incurred by performance of unaccustomed resistance-type exercise, may only elicit adaptive cell signaling responses, where as traumatic necrotizing injury brought on by blunt trauma or laceration may elicit maladaptive responses such as fibrosis and permanent functional impairment (15, 25, 33, 48, 59). Muscle has a remarkable regenerative capacity that allows it to rapidly adapt to changes in both its pattern of activity and acute injury (23, 39). Muscle injury induces a well-characterized inflammatory response involving the recruitment of immune cells and the activation and proliferation of muscle progenitor
cells (3, 22, 36). Together, both muscle and non-muscle cells work in a tightly controlled manner to repair and regenerate the injured tissue.

The inflammatory response to muscle injury occurs in two primary phases: acute and chronic. The acute phase is a pro-inflammatory event, occurring within the first 48 hours post-injury. It involves the release of chemokines (from the injured muscle) into the blood, recruitment of neutrophils and monocytes and the activation of resident macrophages and resident muscle stem cells (satellite cells) within the injured tissue (4, 20, 102). During this initial period, phagocytic neutrophils and macrophages degrade damaged structures via reactive oxygen and nitrogen species (RONS) production, remove debris, and secrete pro-inflammatory chemokines and cytokines such as MCP-1, IFNγ, TNF-α, IL-6, and IL-1β that promote leukocyte chemotaxis and satellite cell proliferation (77, 101). Together, the pro-oxidant and pro-inflammatory processes during the acute phase are believed to be critical for muscle remodeling. However, they also induce secondary muscle damage, which prolongs regenerative processes during the subsequent chronic phase of inflammation, leading to delayed restoration of muscle strength (20, 47, 93). The chronic phase begins ~48 h post-injury, indicated by the decline in neutrophils and phagocytic macrophages and the rise in a non-phagocytic subgroup of macrophages that release anti-inflammatory cytokines such as IL-1, IL-4, and IL-10 (102). The secretion of these cytokines and other unknown factors into the muscle microenvironment is believed to drive the cessation of pro-inflammatory conditions that promote catabolic processes toward anti-inflammatory conditions that promote satellite differentiation and muscle repair.
There is a general consensus in the field that targeting specific acute phase degenerative processes that contribute to secondary damage has been a widely sought after strategy to accelerate restoration of muscle strength following acute injury (6, 16, 63). Numerous signaling molecules and pathways have been demonstrated to play important roles in muscle repair and regeneration (13, 85, 102, 105). However, few studies have demonstrated significant benefits following physical or nutritional interventions geared to reduce secondary damage (76) or accelerate strength-recovery (17, 104), supporting the contention that little is known about the underlying mechanisms that drive muscle adaptation to strenuous exercise and injury.

The purpose of this literature review is to summarize the existing data regarding skeletal muscle damage that is induced by exercise and/or other traumatic injury. Special emphasis is placed on specific cellular and molecular events that regulate the inflammatory and regenerative processes involved in the restoration of muscle function. I begin with a detailed account of the characteristics of exercise-induced muscle damage (EIMD) and then discuss the roles of muscle and non-muscle cells during muscle inflammation and repair. This is followed by a review of the nuclear factor-kappa beta (NF-κB) family of transcription factors. Next, the evidence regarding the efficacy of nutritional interventions designed to improve inflammation and muscle recovery is reviewed. Chapter II closes with a summary of the key concepts and identification of the critical gaps in the literature that I addressed in Dissertation Studies II.
Exercise-induced muscle damage

Eccentrically-biased exercise is well known to cause ultrastructural damage to skeletal muscle fibers (24, 26, 44, 59), which can impair muscle function and increase the susceptibility of the damaged muscles to more severe injury during the days following the initial event (82). This exercise-induced muscle damage (EIMD) is characterized by decrements in muscle strength and increases in soreness, swelling, and blood-levels of muscle-specific enzymes like creatine kinase (CK) (11, 14, 15, 68). Direct observations of EIMD in humans have revealed that eccentrically-biased exercise produces changes in sarcomere architecture (streaming and bisection of the Z-lines) that attributed to the repeated stretching of the muscle during its contractile phase (24, 25). The initial mechanical disruption of sarcomeres can then spread to adjacent regions within the muscle fiber including the sarcoplasmic reticulum and sarcolemma. In conjunction with myofibrilar damage, eccentric exercise induces transient excitation-contraction coupling failure resulting in diminished force generating capacity and elevated intracellular Ca²⁺ concentrations. Increased cytosolic Ca²⁺ levels rapidly activate Ca²⁺-dependent proteases involved in the degradation and remodeling of contractile proteins (75, 82). Furthermore, EIMD initiates a tightly regulated inflammatory response that is accompanied by secondary muscle damage and regeneration (13, 21, 82).

EIMD has become a widely used model to induce and study acute muscle injury, inflammation, and repair in both animal models and humans (13, 20, 45, 48, 67, 74, 80). Although the characteristics of muscle injury and repair are well documented in the aforementioned studies, the mechanisms that drive the inflammatory response at the cellular and molecular levels are largely unknown. Determining how the immune system

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regulates muscle inflammation and repair is essential for developing strategies to reduce the negative consequences of muscle injury and improve recovery of muscle function. Evidence regarding the role of immune cells in muscle inflammation and repair will be addressed in the following sections.

**Myogenesis and muscle regeneration**

Myogenesis is characterized by the fusion of mononucleated precursor cells, myoblasts, or satellite cells, to form a syncytial muscle fiber. (110). Myogenesis is regulated by transcription factors such as myogenin, MyoD, and Myf5, which direct myoblasts to exit the cell cycle and fuse together to form multinucleated myotubes (88). Myogenesis typically refers to the early stages of muscle development; however, myogenesis is largely recapitulated in adult muscle regeneration after injury (109). In adult skeletal muscle, muscle stem cells are typically referred to as satellite cells or muscle precursor cells. Muscle injury induces a regenerative response by skeletal muscle cells which involves the activation and proliferation of the muscle-resident satellite cell pool, followed by satellite cell differentiation and fusion with injured myofibers (99, 110). In adult muscle tissue, the available satellite cell pool comprises approximately five percent of the total number of muscle cells, which is approximately nine times less than the number of satellite cells present during embryonic development (109). This difference in satellite cell availability is also apparent in typical in vitro models of isolated cultures of muscle cells, such as the C2C12 murine muscle cell line used for this Dissertation. Therefore, in vitro muscle injury models consisting of mature myotubes and satellite cells
have an enhanced regenerative capacity, relative to the regenerative capacity of intact adult muscle tissue.

**The role of immune cells in muscle inflammation and injury repair**

A variety of myeloid and non-myeloid cell types reside within muscle, or are recruited to muscle tissue following injury. Inflammatory cells are estimated to be present in regenerative muscle at concentrations beyond 100,000 inflammatory cells/mm³ (113). These cells are activated by soluble molecules such as chemokines and cytokines that are transiently released from the damaged muscle into the microenvironment and the systemic circulation. In response to these muscle-derived molecules, immune cells (i.e. neutrophils and macrophages) secrete cytokines, chemokines, and RONS that regulate the function of other immune cells and affect the viability and regenerative capacity of muscle cells within the injured tissue (101). Furthermore, neutrophils and macrophages are well known to induce secondary muscle damage through their phagocytic activities (78, 80, 86), which are believed to contribute to prolonged muscle soreness, swelling, and strength-loss for several days following acute muscle damage (12, 13, 75, 77, 83). Thus, the communication between the damaged muscle tissue and inflammatory cells contributes to the magnitude and kinetics of the inflammatory response.

Several types of immune cells play distinct physiological roles in systemic and local inflammatory responses (31). However, the precise mechanisms by which they regulate inflammation and muscle remodeling are not well understood. In this section, evidence on the functional roles of neutrophils, monocytes, and macrophages in the inflammatory response to acute muscle damage is presented. Furthermore, the
communication between these immune cells and muscle cells is discussed with a particular emphasis on the role of macrophages.

The activation, invasion, and accumulation of immune cells in damaged muscle follow two temporally distinct events. The acute inflammatory response, characterized by the invasion of neutrophils, monocytes, and activation of phagocytic macrophages, and amplification of the initial injury occurs in the first ~2 days after muscle injury. This is followed by the chronic inflammatory response, characterized by the decline in neutrophils and phagocytic macrophages and the subsequent accumulation of non-phagocytic macrophages. These non-phagocytic macrophages secrete anti-inflammatory cytokines, such as IL-4 and IL-10 into the tissue microenvironment, promoting muscle repair processes that begin ~2 days post-injury, peak after ~4 days, and continue for several days thereafter.

**Neutrophils**

In response to acute injury, skeletal muscle tissue immediately secretes acute-phase pro-inflammatory chemokines and cytokines, such as MCP-1, TNFα and IFNγ, which drive the innate immune response (62). These (and other) cytokines and chemokines promote chemotaxis of circulating neutrophils and monocytes, which then infiltrate the damaged muscle tissue, activate and replenish resident macrophages, and contribute to early muscle remodeling processes (31, 52). Eccentric exercise, reloading of skeletal muscle after unloading, and traumatic injury are all well documented to induce the accumulation of neutrophils (22, 41, 70, 80). Neutrophils are the first phagocytes to invade injured muscle tissue, and their accumulation peaks ~6 h post-injury. Neutrophils
primarily contribute to muscle remodeling by releasing oxidative bursts of myeloperoxidase (MPO) and RONS and engulfing debris. In addition, neutrophils can also directly activate resident macrophages, thereby promoting further muscle cell lysis (61). The release of MPO induces resident macrophages to differentiate into the M1 phagocytic phenotype, as demonstrated by the expression of the cell surface antigen CD68. M1 macrophages release nitric oxide (NO) via the inducible nitric oxide synthase (iNOS) and lyse muscle cells (61). Furthermore, the oxidized LDL from lysed muscle cells binds to the CD68 cell surface receptor, which stimulates M1 macrophage-induced myofiber lysis during the acute inflammatory phase. Thus, neutrophils, and their interaction with macrophages, appear to play important pro-inflammatory and pro-oxidant roles during the acute phase of muscle inflammation.

**Monocytes**

Monocytes are important macrophage progenitor cells that originate from bone marrow, and they are then recruited to inflamed/injured tissues from peripheral blood via inflammatory mediators such as MCP-1(also known as CCL2) (31, 35, 45). When monocytes enter inflamed/injured target tissues, they can differentiate into pro- or anti-inflammatory macrophage phenotypes depending on the specific tissue microenvironment. Circulating monocytes are a heterogeneous population, typically categorized by their expression of a variety of cell surface receptors and antigens (see (28) for review).

The expression of several cell surface receptors found on inflammatory monocytes have been identified to be conserved across humans and rodents, including
(but not limited to): the MCP-1 receptor CCR2; and CD11b and CD14 antigens (31, 106). Two primary subsets of monocytes occur in the blood of humans and mice. The first group, identified as CD14$^{hi}$CD16$^{-}$ (human) and CX3CR1$^{hi}$/Ly-6C$^{hi}$ (mouse) are recruited during acute inflammation (97). The second subset of monocytes identified as CD14$^{lo}$CD16$^{+}$ (human) and CX3CR1$^{hi}$/Ly-6C$^{-}$ (mouse), are recruited in smaller numbers during acute inflammation, but they primarily serve as progenitors for tissue-resident macrophages (28, 45). Although these subpopulations of monocytes appear to have relatively distinct functions, it is not known if they are exclusively recruited to specific tissues (e.g. muscle, intestine, or spinal cord) or if their fates can be altered in response to local stimuli. In the case of muscle injury, evidence suggests that circulating monocytes contribute to the inflammatory response to muscle damage by replenishing resident macrophage populations (3, 45).

Using a mouse model of toxin-induced muscle injury, Arnold et al. (3) demonstrated that injured muscle recruited only the CX3CR1$^{lo}$/Ly6C$^{+}$ monocytes from the blood that exhibited a pro-inflammatory phenotype, and in vivo depletion of circulating monocytes at the time of injury completely prevented muscle regeneration. Then, within the damaged muscle, pro-inflammatory monocytes transitioned to the CX3CR1$^{hi}$/Ly6C$^{-}$ anti-inflammatory phenotype that differentiated into mature anti-inflammatory macrophages (3). This study suggests that in response to toxin-induced muscle injury, recruitment of pro-inflammatory monocytes is critical for muscle regeneration, and once in the muscle, monocytes shift their phenotype and differentiate into anti-inflammatory macrophages that promote muscle repair.
Modulating the processes of either monocyte recruitment macrophage differentiation in damaged muscle tissue may be a viable strategy to attenuate macrophage-induced secondary damage and accelerate recovery of muscle function. However, it is currently unclear if monocyte differentiation in response to inflammatory signals released from mechanically-injured skeletal muscle is similar to toxin-induced muscle injury. Both monocytes and macrophages are known to be highly responsive to chemical signals such as cardiotoxin (28, 31). Therefore, using a toxin-free model of muscle injury may be necessary to determine the role of endogenous signaling molecules in monocyte/macrophone differentiation and communication with injured muscle cells.

**Macrophages**

Macrophages are a diverse set of immune cells that can either cause further damage to muscle tissue, or promote muscle repair, depending on the macrophage phenotype. These cells are sensitive to physical and biochemical changes in their microenvironment and can rapidly shift their physiological function in response to pro- and anti-inflammatory signals (108). Typically, macrophages are believed to play a predominantly beneficial role in muscle repair after injury or reloading and their invasion into damaged muscle tissue is observed in parallel with several tissue repair processes (95, 108).

During the acute inflammatory phase, phagocytic M1 macrophages promote muscle remodeling through NO-dependent phagocytosis and cell lysis, secrete inflammatory mediators such as MCP-1 and IFNγ, as well as other unknown factors that stimulate satellite cell proliferation (102). Work by Meszaros et al. (52) demonstrated that
macrophages can induce apoptosis in neutrophils, which in turn may contribute to attenuated neutrophil-mediated secondary damage to injured muscle tissue. However, the phagocytic subgroup of macrophages designated as M1 has also been observed to lyse muscle cells and contribute to secondary muscle damage (61). These M1 macrophages are the first to invade or become activated in damaged muscle, and are typically activated by neutrophils during the acute phase of inflammation (49). For example, M1 macrophages exhibit strong oxidative burst activity and can injure muscle cells, both in vitro and in vivo, by producing cytotoxic levels of nitric oxide (NO) via the inducible nitric oxide synthase (iNOS) (61). In addition, neutrophils potentiate the activation of M1 macrophages through the release of myeloperoxidase (MPO), which oxidizes low density lipoproteins on damaged muscle cells. In turn, oxidized LDL in the muscle microenvironment ligates the CD68 receptor of resident M1 macrophages, inducing NO release, muscle cell lysis, and production of pro-inflammatory cytokines (84). Furthermore, these macrophages can enhance the cytolytic capacity of neutrophils (61), and depletion of M1 macrophages from inflamed muscle reduces membrane damage (113). Those studies demonstrate that macrophages contribute to pro-inflammatory conditions that promote muscle remodeling, phagocytic activity of neutrophils, and myoblast proliferation during the acute inflammatory response to muscle injury.

In addition to their pro-inflammatory and phagocytic functions, macrophages can also shift their phenotype from M1 to the anti-inflammatory M2 phenotype that has been shown to attenuate neutrophil-mediated muscle damage and promote muscle repair (3, 86, 108). The phenotype shift from M1 to M2 is important because M2 macrophages, which invade damaged muscle during the chronic phase of inflammation, promote
satellite cell differentiation, fusion, and muscle growth (50). During the chronic inflammatory phase, M2 macrophages release anti-inflammatory cytokines such as TGF-β, IL-4, and IL-10, which contribute to the deactivation of M1 macrophages and stimulate differentiation and fusion of myoblasts with existing muscle fibers (3). In addition, phagocytosis of muscle cell debris by M1 macrophages induces the phenotype transition from M1 toward the M2 phenotype, indicated by the release of TGF-β (3). These data suggest that individual subpopulations of macrophages exist within skeletal muscle tissue and that M1 macrophages can shift toward an M2 phenotype after phagocytosing debris.

The transition of macrophages from M1 to M2 is believed to be a critical event for the normal progression of muscle remodeling and regeneration (3, 108). Moreover, failure to transition from M1 to M2 arrests muscle regenerative processes, such as satellite cell differentiation and fusion with muscle fibers which prolongs the resolution of muscle injury (102). However, the underlying mechanisms that contribute to this transition are currently unknown and represent a critical gap in the field. Gaining an understanding of the molecular signaling that drives this process is important for developing targeted strategies to modulate muscle inflammation and facilitate recovery from injury.

In summary, immune cell populations contribute to degeneration and removal of damaged/necrotic structures and subsequently promote muscle regeneration and growth. Immune cells influence the time course of muscle repair, as well as the regenerative capacity of skeletal muscle cells by secreting chemokines and inflammatory cytokines into the tissue microenvironment. Furthermore, these soluble molecules influence the
function of and communication between muscle and non-muscle cell lineages within the injured tissue.

**Transcription factors regulate skeletal muscle stress responses**

Transcription factors are important members of cell signaling pathways that propagate extrinsic signals induced by chemokine and cytokine ligand binding, from the cytosol to the nucleus where they directly regulate gene expression. Typically, transcription factors are bound by proteins in the cytosol which inhibit their activity until they are activated by upstream effectors, such as protein kinases. Once activated, they are released from their inhibitor complexes and translocate to the cell nucleus where they bind DNA in a sequence-specific manner and promote the expression of target genes. Many transcription factors have been shown to play important roles in muscle function and responses to stress. The evidence for the specific family of transcription factors involved in muscle inflammation and regeneration after injury is discussed below.

**The roles of nuclear factor kappa-beta (NF-κB)/Rel family of transcription factors in myogenesis, regeneration, and inflammation of skeletal muscle**

The NF-κB family of transcription factors is composed of five protein subunits: p50, p65, c-Rel, Rel B, and p52. NF-κB can be activated by multiple signaling pathways, such as p38 MAPK and ERK 1/2, as well by growth factors (e.g. IGF-1 and TGF-β), inflammatory cytokines such as TNFα, and RONS (2, 5, 57). Collectively, NF-κB regulates the expression of over 150 genes, including those encoding chemokines (i.e.
MCP-1), inflammatory cytokines (i.e. TNFα and IL-6), and antioxidant defense enzymes (i.e. SOD and iNOS) (42, 57).

There are two distinct pathways by which NF-κB is regulated: the classical pathway represented by the p65 subunit, and alternative pathway represented by the RelB subunit. In skeletal muscle, the classical NF-κB pathway, typically represented by the p65 subunit of the p65/p50 heterodimer complex, is a well-studied regulator of skeletal myogenesis (4, 5, 109), and a primary focus of Study II of this dissertation. The transactivation status of the classical NF-κB pathway has been well-established to play important roles in developmental myogenesis and adult skeletal muscle inflammation and regeneration after injury. Recently, Bakkar et al. (5) observed that regulation of myogenesis is dependent on the transcriptional activity of p65, which promotes satellite cell proliferation via activation of cyclin D1 and negatively regulates satellite cell fusion with muscle fibers by silencing differentiation-related genes such as MyoD (32). Furthermore, genetic knockout of p65 in mice stimulates muscle regeneration following cardiotoxicin-induced injury, and provision of pharmacological inhibitors of p65 activation, such as curcumin, a polyphenolic phytonutrient, increases biomarkers of muscle regeneration following freeze-injury in mice (51, 100).

Recent studies of biopsied exercise-induced muscle injury in humans have provided evidence that the classical NF-κB pathway, represented by the p65 subunit, is activated in injured skeletal muscle within 2-3h post-exercise (38, 107). In one of those studies (38), it was suggested that the overall abundance of p65 within muscle cells (i.e. myogenic precursor cells, and differentiated muscle fibers) was relatively low and that much of the increased p65 transactivation was accounted for by expression of p65 in non-
muscle cells resident within the injured tissue. However, other lines of evidence are in
disagreement with the notion that muscle cells express low-levels of NF-κB. Instead, both
the classical and alternative NF-κB pathways have been reported to play indispensable
roles in muscle inflammation, myogenesis, and regeneration; such that genetic
knockdown of either pathway has significant consequences for basic muscle cell
homeostasis, stress signaling, and regenerative responses to injury (5, 32, 51, 100).

The alternative NF-κB pathway, represented by the Rel B sub-unit, was also
shown by Bakkar and colleagues to regulate mitochondrial function and biogenesis-
related processes in healthy myotubes and to rescue unhealthy myotubes from starvation-
induced atrophy and cell death (5). In that study, genetic over-expression of IKKα, an
upstream inducer of alternative NF-κB complex activation and nuclear translocation,
significantly upregulated mitochondrial content and function, and rescued myotubes from
catabolism and cell death (5). Those findings suggest that in cultured skeletal muscle
cells, the alternative NF-κB pathway contributes to muscle cell maintenance and prevents
catabolism under stressful conditions. In theory, this process may be geared toward
increasing the available pool of proliferating satellite during the Th1 phase following
acute injury (5) and muscle hypertrophy in response to exercise training (19). Those
studies demonstrate that both classical and alternative NF-κB pathway signaling are
important regulators of muscle regeneration after injury.

In addition to its regulatory role in muscle regeneration, NF-κB is also a critical
regulator of inflammatory signaling in immune cells, specifically in macrophages.
Several lines of evidence have shown that NF-κB (i.e. p65) directly regulates iNOS
expression in human macrophages, and that inhibition of NF-κB activation
downregulates iNOS expression and activity (58, 60, 71-73). Moreover, Nguyen and Tidball (61) demonstrated that M1 macrophages can induce secondary damage to muscle cells *in vitro* through iNOS-dependent release of NO. Those studies suggest that targeting NF-κB-dependent iNOS expression in macrophages may be a viable strategy to modulate secondary muscle damage and promote muscle recovery following injury.

**Therapeutic strategies to modulate inflammation and improve recovery**

Pharmacological strategies designed to reduce macrophage-induced inflammation in non-muscle cell lines have shown promising results (58, 71-73). For example, Overman et al. (73) recently demonstrated *in vitro* that administration of the anti-inflammatory compound quercetin inhibited the expression of the acute phase cytokines TNF-α, IFN-γ, IL-6, and IL-1β in human macrophages. As a result, quercetin decreased induction of TNF-α, IFN-γ, and MCP-1 in human adipocytes treated with the culture medium from quercetin-treated macrophages. Those data, and the work of others (58, 71), also showed that quercetin inhibited the pro-inflammatory activity of macrophages and the induction of MCP-1 in other cell populations via inhibition of NF-κB activity. The effect of quercetin on MCP-1 expression in non-muscle cells is particularly compelling because MCP-1 has also been shown to play a primary role in the inflammatory response to muscle damage (36, 45, 115). MCP-1 is regarded as an important chemokine that regulates the recruitment of circulating monocytes (3, 35, 89, 94), activation of satellite cells and macrophages following acute muscle injury (36, 45, 46, 90, 115). For instance, Hubal et al. (36) observed colocalization of MCP-1 in macrophages and satellite cells within injured human muscle tissue, and Yahaoui et al.
(115) demonstrated in vitro that recombinant MCP-1 treatment accelerated wound closure by stimulating post-injury myoblast proliferation, contributing to muscle repair. MCP-1 appears to be a primary signaling molecule that regulates communication between myoblasts, monocytes, and macrophages and contributes to muscle regeneration. Therefore, it is reasonable to hypothesize that inhibition of NF-κB and modulation of MCP-1 secretion, might reduce macrophage-induced secondary damage, and in turn, reduce local muscle inflammation following exercise-induced muscle injury.

Summary

The inflammatory response to acute muscle injury is a tightly controlled process that has been well characterized over the past several decades. The initial damage to the muscle cell membrane is believed to trigger the release of chemoattractant molecules such as MCP-1, which in turn rapidly induces the recruitment and activation of immune cells to the injured tissue. Among the many leukocyte cell types, monocytes and macrophages play particularly important (and complex) roles in muscle remodeling and regeneration. However, it is currently unknown which inflammatory molecules have the greatest impact on monocyte/macrophage recruitment and activation. Furthermore, it is unknown if de novo transcription of chemokines and cytokines via NF-κB activation is required to drive muscle inflammation and regeneration after injury. Therefore, determining the molecular mechanisms of communication between injured skeletal muscle cells and immune cells will reveal novel evidence that can be used to develop therapeutic strategies to accelerate recovery from injury.
CHAPTER III

EFFECTS OF QUERCETIN SUPPLEMENTATION ON MARKERS OF MUSCLE DAMAGE AND INFLAMMATION AFTER ECCENTRIC EXERCISE

Abstract

The flavonoid quercetin is purported to have potent antioxidant and anti-inflammatory properties. This study examined if quercetin supplementation attenuates indicators of exercise-induced muscle damage in a double-blind laboratory study. Thirty healthy subjects were randomized to quercetin (QU) or placebo (PL) supplementation and performed 2 separate sessions of 24 eccentric contractions of the elbow flexors. Muscle strength, soreness, resting arm angle, upper arm swelling, serum creatine kinase (CK) activity, plasma quercetin (PQ), interleukin-6 (IL-6), and C-reactive protein (CRP) were assessed before and for 5 d after exercise. Subjects then ingested nutrition bars containing 1,000 mg/d QU or PL for 7 d before and 5 d after the second exercise session, using the opposite arm. PQ reached 202 ± 52 ng/ml after 7 d of supplementation and remained elevated during the 5-d post-exercise recovery period (p<.05). Subjects experienced strength loss (peak=47%), muscle soreness (peak=39 ± 6 mm), reduced arm angle (−7° ± 1°), CK elevations (peak=3,307 ± 1,481 U/L), and arm swelling (peak=11 ± 2 mm; p<.0001), indicating muscle damage and inflammation; however, differences between treatments were not detected. Eccentric exercise did not alter plasma IL-6 (peak=1.9 pg/ml) or CRP (peak=1.6 mg/L) relative to baseline or by treatment. QU supplementation had no effect on markers of muscle damage or inflammation after eccentric exercise of the elbow flexors.

Keywords: flavonoid, muscle soreness, DOMS, strength loss
Introduction

Eccentrically biased exercise is well known to cause damage to skeletal-muscle fibers, characterized indirectly by decrements in strength, development of muscle soreness, swelling, and increased blood levels of muscle specific proteins such as creatine kinase (CK) in the days after the exercise bout (11). Moreover, exercise-induced muscle damage (EIMD) initiates an inflammatory response associated with secondary muscle damage and remodeling (13).

The inflammatory response to EIMD occurs in two primary phases. During the acute phase, both neutrophils and phagocytic macrophages can release reactive oxygen and nitrogen species and remove debris by phagocytosis (102). In addition, cytokines and byproducts of reactive oxygen and nitrogen species from the injured muscle are released into the blood, contributing to low-grade systemic inflammation and oxidative stress, elevating blood C-reactive protein (CRP) levels (33), and altering glutathione redox status (29). Together, the pro-inflammatory and pro-oxidant processes can induce secondary damage to the injured tissue, prolonging repair and regenerative processes during the subsequent chronic inflammatory phase, characterized by restoration of muscle strength and resolution of inflammation (76, 93).

Supplementation with antioxidant and anti-inflammatory nutrients can, in some cases, attenuate oxidative stress (29, 30), inflammation (79), and muscle soreness (6) and improve strength recovery (17, 104) after eccentric exercise. Quercetin is a flavonol-type polyphenol that has been extensively studied for its antioxidant and anti-inflammatory properties (1, 65, 73). Recently, Overman et al. reported that quercetin attenuated expression of inflammatory cytokine TNF-α, IFN-γ, IL-6, and IL-1β transcripts in
cultured human macrophages, which are known contributors to secondary muscle damage (102). Those data suggest that polyphenols (i.e., quercetin) can modulate acute-phase inflammatory mediators. Phillips et al. (79) showed that consumption of a mixed dietary supplement containing 300 mg mixed tocopherols, 800 mg docosohexaenoate, and 300 mg polyphenols (100 mg hersperatin and 200 mg quercetin) for 14 days before eccentric exercise of the elbow flexors significantly attenuated changes in IL-6 and CRP levels at 3 days post-exercise relative to placebo. Therefore, we tested the hypothesis that a mixed supplement containing 1,000 mg/d quercetin would attenuate strength loss and inflammation after strenuous eccentric exercise of the elbow flexors in young, healthy men and women.

**Methods**

**Subjects**

Thirty subjects age 18–25 years were recruited from the University of Massachusetts Amherst campus and the local community. After subjects provided written consent for participation (Visit 1), testing and analyses of blood measures (except quercetin and CK) were conducted. Subjects were sedentary to recreationally active, naïve to resistance training and resistance-type activities of the upper extremities for 6 months before participation, negative (by self-report) for family history of and current musculoskeletal or metabolic impairments, and not taking dietary supplements. Subjects refrained from resistance-type activities and use of dietary supplements and over-the-counter and prescription anti-inflammatory medications.
Study Design

The study was conducted in a randomized, double-blind, placebo-controlled manner and is shown in Figure 3.1. At Visit 1, they were provided a list of quercetin-containing foods and agreed to refrain from excessive consumption of them (10) for 3 days before and during the study.

Figure 3.1. Study design and timeline. The study consisted of 15 visits (V1–15) over 3 phases. Phase 1: performed eccentric contractions (EC) with 1 arm (V3), measures taken at baseline (V2–3), immediately post-exercise (V3), and every 24 hr for 120 hr thereafter (V4–8). Phase 2: ingested quercetin or placebo bars at ~8 a.m. and ~8 p.m. each day for 7 days. Phase 3: continued supplementation (V9–15), EC of the contralateral arm (V10), measures taken at baseline (V9–10), immediately post-exercise (V10), and every 24 hr for 120 hr thereafter (V11–15). Criterion measures: isometric and isokinetic strength at 60°/s and 180°/s (Strength), muscle soreness (Soreness), resting arm angle (RAA), arm circumference (AC). Biological markers: plasma quercetin (PQ), interleukin-6 (IL-6), C-reactive protein (CRP), and serum creatine kinase (CK).

Subjects reported to the laboratory in a fasted state (≥10 hr) and rested for 10–15 min before data collection each day. They were randomly assigned in a permuted block design to perform exercise Bout 1 with either the non-dominant or the dominant arm and
take a quercetin- or placebo-containing supplement. Arm dominance was determined by subject self-report, and subjects were balanced for arm dominance as evenly as possible within groups. Fifteen subjects received placebo, and 15 subjects received the quercetin-fortified supplement. Subjects performed two bouts of 24 maximal eccentric contractions of the elbow flexors using a modified preacher-curl bench as reported previously (67). Bout 1 was performed in Phase 1, and Bout 2 was performed with the contralateral arm in Phase 3. During supplementation, subjects received either 1,000 mg/d quercetin aglycone (Merck, SA Brazil) via First Strike nutrition bars (Natick Soldier Center, Natick MA) or placebo First Strike bars. Each First Strike bar also contained 10 mg vitamin C and 7 mg total tocopherols, which resulted in daily doses of 20 mg vitamin C and 14 mg tocopherols. Six days after Bout 1 (i.e., 24 hr after Visit 8), subjects ingested one bar, twice daily at 12-hr intervals, for 7 consecutive days. The dosage regimen was based on our previous human pharmacokinetic studies (40) in which plasma quercetin (PQ) was undetectable at baseline via high-performance liquid chromatography (minimum limit of detection of 3 ng/ml), followed by significant (p<.05) peak increases (620.7 ng/ml) in PQ after participants (N=18) ingested quercetin-fortified First Strike bars. Thus, quercetin levels were expected to be negligible in the blood, unless quercetin was ingested via supplementation. Therefore, due to the prohibitive cost of analysis, PQ was not determined from subjects in the placebo group in the current study, which we highlight as a limitation. Compliance was verified by the investigator by counting empty supplement wrappers when subjects returned for Phase 3. Subjects ingested the quercetin or placebo supplement immediately after all measures were collected on each visit and were provided a second bar to ingest 12 hr later.
Experimental Measures

Isometric and isokinetic strength were assessed on an isokinetic dynamometer (Biodex System 3, Biodex, Shirley, NY). Subjects were seated on the Biodex with the elbow fixed at 90° flexion, and their body position, relative to the lever arm, was documented and maintained throughout the study. Three isometric strength trials (3 s/trial) with 1-min rests between trials, 12 consecutive isokinetic contractions at 60°/s and at 180°/s, muscle soreness, relaxed arm angle, and upper arm circumference were assessed daily.

Blood samples were obtained after subjects rested in the laboratory for 10–15 min and fasted overnight for ≥10 hr. Samples for PQ, CRP, and IL-6 were collected in K2 EDTA Vacutainers (BD Biosciences, USA) and centrifuged at 3,000 g at room temperature (quercetin) or at 4 °C (CRP and IL-6). Samples for CK enzyme activity were collected in serum Vacutainers (BD Biosciences), clotted for 15 min, and centrifuged at 3,000 g at room temperature. Aliquots were stored at −80 °C for subsequent analyses.

Analyses of PQ were performed at Rutgers University via high-performance liquid chromatography, with a minimum detectable [PQ] of 3 ng/ml. CRP was assessed using an enzyme-linked immunosorbent assay (ELISA; R&D Systems Inc., Minneapolis, MN) with a minimum detectable [CRP] of 0.010 ng/ml, as per manufacturer’s protocol.

IL-6 was assessed via high-sensitivity ELISA (R&D Systems Inc.) with a minimum detectable [IL-6] of 0.039 pg/ml, as per manufacturer’s protocol. CK activity was assessed at Holyoke Hospital, Holyoke, MA.
Statistical Analyses

Data were analyzed using the Statistical Analysis Software (SAS) package, (V9.2; SAS Institute, Cary, NC). Reliability of pre-exercise measurements was assessed using an intra-class R analysis. For isometric and isokinetic strength, arm angle, and arm circumference, the R values were .94, .95, .93, and .94, respectively. A repeated-measures ANOVA with a grouping factor (placebo vs. quercetin) was used to compare measures before and after supplementation (Phase 1 vs. Phase 3). Arm circumference and CK data were not normally distributed and were log-transformed before analyses. Significance was set at p<0.05.

Results

Subject Characteristics

All subjects completed both exercise sessions and were compliant with the inclusion and exclusion criteria and supplementation requirements. Subject characteristics for the entire cohort are shown in Table 3.1. Data represent means ± SD. There were no significant differences between the placebo and quercetin groups.

Table 3.1. Physical characteristics for all subjects (N=30), partitioned by supplement group and sex.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Placebo (N = 15)</th>
<th>Quercetin (N = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Men (N=8)</td>
<td>Women (N=7)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>19.5 ± 1.1</td>
<td>19.6 ± 1.3</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.78 ± 0.1</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>76.0 ± 17.2</td>
<td>60.0 ± 9.6</td>
</tr>
<tr>
<td>Body-mass index (kg/m2)</td>
<td>24.0 ± 5.1</td>
<td>22.5 ± 2.1</td>
</tr>
</tbody>
</table>
Isometric and Isokinetic Strength

Eccentric exercise induced significant (p<0.01) isometric strength loss (Figure 3.2.) immediately post-exercise that returned toward baseline values over the next 120 hr. A significant (p<0.01) Phase × Time interaction was observed, with no significant Group × Phase or Group × Time interactions, indicating that the placebo and quercetin groups responded similarly. Isokinetic strength at 60°/s (Figure 3.3.) and 180°/s (data not shown) was significantly (p<0.01) decreased immediately post-exercise and returned toward baseline values over the next 120 hr. However, no significant main effects of group or phase or interactions were observed, indicating that quercetin did not attenuate muscle-strength loss or facilitate recovery of baseline strength.

Figure 3.2. Isometric strength loss pre- vs. post-supplementation. Effects of (A) placebo (N=15) and (B) quercetin (N=15) on isometric peak torque at baseline (BSLN), immediately post-exercise (IMM. POST), and 24–120 hr thereafter, M ± SEM. *Significantly different from BSLN (p<0.01).
Figure 3.3. Isokinetic strength loss pre- vs. post-supplementation. Effects of (A) placebo (N=15) and (B) quercetin (N=15) on isokinetic peak torque at 60°/s at baseline (BSLN), immediately post-exercise (IMM. POST), and 24–120 hr thereafter, M ± SEM. *Significantly different from BSLN (p<0.01).

Muscle Soreness

Soreness was assessed using a 100-mm visual analog scale that indicates no soreness on the far left (0 mm) of the scale and unbearable pain on the far right (100 mm; Figure 3.4.). Subjects made a single vertical mark on the scale that corresponded to their soreness level. A significant (p<0.01) effect of time was observed, indicating a significant increase in muscle soreness that peaked within ~48 hr post-exercise and returned toward baseline values by 120 hr. There were no significant main effects of group or phase, nor were any significant interactions observed, indicating that quercetin supplementation did not attenuate exercise-induced muscle soreness.

Figure 3.4. Changes in perceived muscle soreness pre- vs. post-supplementation. Effects of (A) placebo (N=15) and (B) quercetin (N=15) on changes in muscle soreness at baseline (BSLN) and 24–120 hr post-exercise, M ± SEM. *Significantly different from BSLN (p<.01).
Swelling

Eccentric exercise produced a significant (p<.01) increase in upper arm circumference, indicating local muscle swelling within 24 hr after the exercise that peaked at ~120 hr (Figure 3.5). No significant main effects of group or phase or interactions were observed, indicating a similar response between the placebo and quercetin groups. There appeared to be a trend (p=0.14) toward a significant attenuation in swelling (Bout 1 vs. Bout 2) in the quercetin group at ~120 hr (Figure 3.5.B), which may suggest an effect of quercetin on swelling at this late time point. However, this trend was not statistically significant.

Figure 3.5. Changes in upper-arm circumference pre- vs. post-supplementation. Effects of (A) placebo (N=15) and (B) quercetin (N=15) on changes (Δ) in circumference of the mid-biceps region at baseline (BSLN) and 24–120 hr post-exercise, M ± SE. *Significantly different from BSLN (p<0.01).

Serum CK

Serum CK activity increased significantly (p<0.05) within 24 hr and remained elevated at 120 hr post-exercise (Figure 3.6.). There were no significant main effects of group or phase on CK, nor were any significant interactions observed among the main effects, indicating no differences within or between placebo and quercetin groups.
Figure 3.6. Changes in serum creatine kinase (CK) activity pre- vs. post-supplementation. Effects of (A) placebo (N=15) and (B) quercetin (N=15) on CK at baseline (BLN) and 24–120 hr post-exercise, M ± SEM. *Significantly different from BLN (p<0.05).

Resting Arm Angle

A significant (p<0.01) decrease in arm angle was observed immediately post-exercise, which returned to baseline at 120 hr. However, there were no significant main effects of group or interactions observed, indicating that the placebo and quercetin groups responded similarly (data not shown).

PQ, IL-6, and Serum CRP

Table 3.2. presents the data for PQ in ng/ml, IL-6 in pg/ml, and CRP in mg/L for the placebo and quercetin groups. The mean PQ levels ranged from ~15 to 17 ng/ml during Phase 1 and significantly (p<.05) peaked at ~202 ± 52 ng/ml among subjects in the quercetin group after 7 days of supplementation (Phase 3, Visit 9) with quercetin-fortified (1,000 mg/d) First Strike bars (Table 3.2.). During Phase 3, mean PQ remained significantly (p<.05) elevated (relative to pre-supplement levels) at ~103–202 ng/ml each day for 5 days post-exercise. No significant group, phase, or time effects on these markers of systemic inflammation were observed, nor were any significant interactions.
Neither the exercise protocol nor quercetin supplementation altered blood IL-6 or CRP concentrations.

Table 3.2. Effects of Supplementation on Blood Markers of Plasma Quercetin and Inflammation. Data represent means ± SEM. *Significantly different from BSLN (p<0.05).

<table>
<thead>
<tr>
<th>Blood marker</th>
<th>Group</th>
<th>Pre-supplement</th>
<th>Post-supplement</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Baseline</td>
<td>Peak</td>
</tr>
<tr>
<td>Plasma quercetin (ng/ml)</td>
<td>quercetin</td>
<td>14.70 ± 3.93</td>
<td>16.96 ± 4.4 (4 days)</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>placebo</td>
<td>1.05 ± 0.23</td>
<td>1.91 ± 0.76 (3 days)</td>
</tr>
<tr>
<td></td>
<td>quercetin</td>
<td>0.66 ± 0.16</td>
<td>0.98 ± 0.26 (3 days)</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>placebo</td>
<td>0.95 ± 0.28</td>
<td>1.60 ± 0.47 (4 days)</td>
</tr>
<tr>
<td></td>
<td>quercetin</td>
<td>0.93 ± 0.14</td>
<td>1.48 ± 0.55 (5 days)</td>
</tr>
</tbody>
</table>

Discussion

The aim of this study was to assess the effects of quercetin supplementation on markers of muscle damage and systemic inflammation after strenuous eccentric exercise of the elbow flexors. Each exercise bout induced significant decrements in isometric and isokinetic strength, development of muscle soreness, elevation in serum CK, and development of local muscle swelling, patterns typical to previous investigations of EIMD (53, 67, 104). However, we demonstrated no effect of quercetin supplementation on the aforementioned markers of EIMD and no effect of quercetin or eccentric exercise on biological markers of systemic inflammation (IL-6 and CRP). Our findings support the recent report by Goldfarb et al. (2011) that a nutritional supplement containing vitamins C and E and polyphenols does not attenuate changes in muscle force or function. Contrary to the report by Phillips et al. (2003), we found no significant increase in IL-6 and CRP, relative to baseline, in blood of fasted participants collected every 24 hr for 5 days post-exercise.
Although some studies have reported changes in IL-6 and CRP at similar time points after eccentric exercise (74, 79), others have not (53, 55, 67, 104). For example, Miles et al. (2008) found that plasma IL-6 significantly (p<0.003) peaked at ~8 hr after eccentric exercise and was not significantly different from baseline at 24 through 120 hr post-exercise, and no significant change in CRP was evident at 24 through 120 hr post-exercise, relative to baseline. Those findings demonstrate that eccentric exercise of the elbow flexors induces a small increase in plasma IL-6 at ~8 hr that returns to baseline by 24 hr post-exercise. Our data corroborate previous reports (53, 67, 104) that eccentric exercise of the elbow flexors, performed at a volume and intensity comparable to that in the current study, does not produce sustained increases in systemic IL-6 and CRP levels lasting up to and beyond 24 hr post-exercise (i.e., assessed at 48, 72, 96, and 120 hr post-exercise).

Seven days of quercetin supplementation (1,000 mg/d) significantly elevated PQ concentrations (~202 ± 52 ng/ml) in blood samples collected from fasted (≥10 hr) subjects, indicating that circulating PQ was detectable at appreciable concentrations at ~10–12 hr post-supplementation. These findings support recent work by Nieman et al. (2009) in which fasted PQ levels were elevated (~250 ng/ml) after 14 days of quercetin supplementation with 1,000 mg/d. Although our dosing regimen effectively maintained elevated PQ levels during supplementation, the supplement was not effective at attenuating changes in markers of EIMD. One possible explanation for these findings is that the biological activity of quercetin found in food is diminished during small-intestinal and hepatic metabolism (43), resulting in decreased potency after absorption into the blood compartment. In the small intestine, quercetin is conjugated to sugar.
moieties to form quercetin-glucuronides and can be further glucuronidated, sulfated, or methylated in the liver (69).

The metabolism of quercetin is believed to substantially lower its bioactivity in vivo. For example, Day, Bao, Morgan, and Williamson (18) suggested that the antioxidant activity of conjugated quercetin is approximately 50% that of the parent quercetin aglycone, which is the most biologically active form. Moreover, quercetin aglycone is less bioavailable in humans than its metabolites such as quercetin-3’-sulfate and quercetin-3-glucuronide (34). Although our data showed that PQ was significantly elevated after oral ingestion, it is possible that the biological activities of the quercetin metabolites present in the blood after oral ingestion are not sufficient to exert protective effects against the negative consequences of eccentrically biased exercise.

Here we used an eccentric-exercise model of the elbow flexors to show that quercetin supplementation had no effect on any commonly used markers of muscle damage and recovery. Although our protocol did not detect an effect of quercetin, two recent studies found that polyphenol supplementation significantly improved muscle-strength recovery after eccentric actions of the elbow flexors (17, 104), demonstrating that the elbow-flexor model is appropriate for detecting the effects of nutritional interventions on recovery of muscle strength after EIMD. Like Connolly et al., we observed comparable decrements in muscle strength immediately post-exercise (−39% vs. −29%) and peak muscle soreness at 48 hr post-exercise (+41% vs. +45%). Thus, we believe that our model was appropriate to detect an effect of quercetin on recovery of muscle strength had it been present.
In young, healthy individuals, quercetin is purported to have mild effects on innate immunity, manifested as a reduced rate of incidence of exercise-induced respiratory illness (64) and altered levels of inflammatory mediators after strenuous aerobic exercise (63, 65). These findings suggest that quercetin may, in some cases, alter the systemic inflammatory response to strenuous aerobic exercise; however, they do not support the notion that quercetin itself improves prolonged aerobic-exercise performance. In addition, Abbey and Rankin (2011) recently showed no effect of quercetin on repeated-sprint performance in college age athletes, which suggests that quercetin also has no effect on physical performance of anaerobic exercise. Therefore, in the context of exercise performance in healthy humans, quercetin supplementation does not appear to enhance performance of aerobic exercise or attenuate decrements in muscle function in response to eccentrically biased resistance exercise, as we have shown here.

Conclusion

Our exercise protocol induced prolonged decrements in isometric and isokinetic peak torque, soreness, CK release into the blood compartment, and muscle swelling. Ingestion of 1,000 mg/day of quercetin delivered in fortified nutrition bars for 7 days before and for 5 days after an acute bout of eccentric contractions of the elbow flexors significantly increased PQ levels. However, no effect of quercetin was observed on any indices of muscle damage or inflammation. Our findings demonstrate that although quercetin is safe for human consumption, it did not attenuate the negative consequences of strenuous eccentrically-biased exercise in this study.
CHAPTER IV
DEVELOPMENT OF THE IN VITRO MUSCLE INJURY MODEL

Introduction

In vitro models of muscle damage have been used previously to induce initial mechanical disruption to cells in culture and examine the mechanisms of muscle repair and regeneration (9, 105, 115). These in vitro models are useful for muscle research because they allow the examination of the muscle cell-specific molecular signaling responses to injury in a tightly controlled system. In contrast, in vivo models using intact tissue from animals or humans affords the opportunity to examine the net response of muscle cells and non-muscle cells such as macrophages, endothelial cells, and pericytes, which are resident in the intact tissue. However, a key limitation of in vivo models is the inability to precisely distinguish the molecular responses of muscle cells (i.e. satellite cells and myotubes) from the molecular responses of non-muscle cells within the intact tissue. Because the overall purpose of dissertation Study II was to determine the roles of NF-κB activation and MCP-1 expression and secretion from muscle cells, I chose to take an in vitro approach to determine these muscle cell-specific molecular responses to injury.

This Chapter documents the experimental design, methods, and preliminary data generated during the development of the in vitro model of muscle injury used in Study II of this dissertation. A scratch-induced injury cell culture model was adapted from the work of Yahaoui et al. (115) with C2C12 myoblasts and the work of Tsivitse et al. (105) with cultured human myotubes. In preparation for this dissertation work, I conducted pilot experiments to replicate the findings from previously validated in vitro injury models (105, 115) and to develop the methods that I would use to test the hypotheses for
Study II. In those studies, the investigators used either isolated cultures of myoblasts (115), or mixed cultures containing both myoblasts and myotubes (105) in their respective muscle injury models. Therefore, I found it necessary to conduct pilot experiments to demonstrate my ability to integrate each of those models into a single in vitro muscle injury model, and to successfully reproduce the findings from those studies. The initial scratch-injury was designed to induce a similar magnitude of damage and time course of wound-healing to what was reported in the previously validated models (105, 115). The data illustrated in Figures 4.1-4.10 below demonstrate that the model I developed for this dissertation work is suitable to induce muscle injury in vitro and study the response of cultured C2C12 myoblasts and differentiated myotubes.

Methods

Myogenic Cell Culture

C2C12 myoblasts [(a mouse satellite cell line; Yaffe and Saxel, 1977 (114)] purchased from American Type Culture Collection (ATCC, Manassas, VA) were cultured in 10cm² sterile polystyrene tissue culture dishes in the presence of growth medium (GM) composed of: Dulbecco’s Modified Eagle Medium (DMEM) (GIBCO, Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Atlanta, GA) and 1% penicillin/streptomycin (GIBCO, Invitrogen Corporation, Carlsbad, CA) at 37°C in the presence of 10% CO₂. Cultures were fed fresh GM every 24 hours (h). When cultures reached ~80% confluency the medium was aspirated and the cells were rinsed once with 1X phosphate buffered saline (PBS), before incubation with 3ml 0.5% trypsin-EDTA (GIBCO, Invitrogen Corporation, Carlsbad,
CA) for 4 minutes (min) at 37°C to lift cells from the bottom of the culture dish prior to transferring them to sterile 15ml conical polystyrene tubes (Fisher Scientific, USA) containing 3ml fresh DM to halt the trypsinization process. Cells were then pelleted by centrifugation at 4000 x g for 90 seconds (s). The supernatant was then aspirated and the cell pellet was resuspended in 2ml fresh GM via 40 cycles of repeat pipetting with a rubber bulb and glass Pasteur pipette. A 40µl aliquot of cell suspension was then diluted into 40µl Trypan Blue dye (GIBCO, Invitrogen Corporation, Carlsbad, CA) and incubated at room temperature for 2 min. Then, 10µl of the Trypan Blue-containing cell suspension was applied to each chamber of a Neubauer hemacytometer and cell viability was determined under a Nikon TMS inverted microscope via the Trypan Blue exclusion method per the manufacturer’s protocol. Cells that excluded Trypan Blue dye were deemed to be alive while those that stained blue were counted as dead and were excluded from the total viable cell count. The cell suspension was then serially diluted in GM and seeded at a standardized density in sterile tissue culture dishes to be used for experiments. Cells were monitored and GM was aspirated and replaced with fresh GM every 24h until the cells reached ~80% confluence. GM was then aspirated and replaced with differentiation medium (DM) composed of: DMEM supplemented with 2% horse serum and 1% penicillin/streptomycin (GIBCO, Invitrogen Corporation, Carlsbad, CA) at 37°C in the presence of 10% CO₂. Cultures were then fed fresh DM every 24h until myoblast cultures differentiated into mature, multi-nucleated myotubes. Myoblasts were allowed to differentiate into multinucleated myotubes for 120h prior to experimentation. During the differentiation period, DM was then aspirated and replaced with fresh DM every 24h. All experiments were performed using cell passages ranging from passage 6 through passage
10. The standard C₂C₁₂ myogenic cell culture procedures are illustrated below in Figure 4.1.

**Figure 4.1.** Standard C₂C₁₂ myogenic cell culture procedures. Myoblasts were thawed and seeded onto 10cm² culture dishes and incubated in growth medium (GM) until they reached ~80% confluency (i.e. until they covered ~80% of the total surface area of the dish). Myoblasts were then induced to differentiate by incubation in reduced-serum (reduced from 10% serum in GM to 2% serum in DM) differentiation medium (DM) for 120h until myotube cultures reached maturity.

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**In vitro muscle injury experimental design & methods**

Myoblasts were cultured in 10cm plates under standard conditions (as described above) with GM containing 10% FBS until they reached ~80% confluency. For myotube experiments, cultures were then switched to DM and myoblasts were allowed to differentiate for 120h until they reached maturity (Figure 4.2.). Mechanical disruption was then used to induce injury to the confluent monolayer of myoblasts or myotubes by tracing a symmetrical grid that was placed under the tissue culture plate with a plastic cell
scraper. The grid consisted of 5 horizontal and 5 vertical lines that created 13mm squares (lines within the black dashed area shown at right, Figure 4.2). The monolayer was “injured” using a sterile gel-loading pipette-tip to generate a continuous and well-delineated area of cellular disruption along the surface of each plate. Samples (200µL out of 10ml total volume) of the culture medium were collected at each time point and immediately frozen at -80°C for subsequent biochemical analyses. Phase contrast images were collected at baseline, immediately post, and 1, 3, 6, 24, 48, and 72h after induction of scratch-injury. Images were then visually inspected using phase-contrast microscopy to qualitatively evaluate changes in the size of each lesion before and after scratch-injury. Within the plate, multiple images of non-overlapping injury areas (i.e. the empty space between confluent cells at the identical region of injury) were collected at each time point. The specific regions of injured and uninjured control myotubes to be imaged were identified randomly (a priori) on the grid. The complete experimental design is shown below in Figure 4.3.
**Figure 4.3.** Experimental design for *in vitro* muscle injury. All experiments were performed independently and 3-6 biological replicates were collected per time point and under each condition. Myotube cultures were scratch-injured. ELISA assays to determine NF-κB/p65 DNA binding activity in nuclear extracts and MCP-1 protein expression in cytosolic extracts and conditioned medium, were conducted by analyzing each biological replicate in duplicate via ELISA assays (described in Chapter V).

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**Blockade of NF-κB transactivation and MCP-1 protein secretion**

A pharmacological approach was used to determine the role of NF-κB in post-injury regeneration. PR39 (R&D Systems, Minneapolis, MN, Catalog #1947) is a porcine-derived peptide inhibitor of NF-κB transactivation. PR39 selectively binds the α7 subunit of the 20S proteasome and blocks IκBα (the cytosolic inhibitor of the NF-κB p65/p50 heterodimer) degradation by the ubiquitin proteasome pathway without disrupting overall proteasome activity (27). PR39 was chosen for this research because it has been shown in various cell lines *in vitro* to suppress NF-κB-dependent gene expression both in cell culture and in two models of acute injury in mice (27). Dose-response experiments were conducted at the outset of dissertation Study II to empirically determine the optimal concentration of PR39, and the treatment-duration of myotube cultures (Figures 4.8.-4.10).
Dissertation Study II Pilot Data

The results from the experiments described above are illustrated in Figures 4.4.-4.10. Figure 4.4. shows the results from injured satellite cell (myoblast) cultures. The results from heterogeneous myotube culture experiments are shown in Figures 4.5.-4.10.

Figure 4.4. Images of scratch-injured myoblast cultures at baseline (BSLN), immediately post-injury (IMM POST), and 24h (24H) post-injury. **Left:** Uninjured field of myoblasts at baseline (BSLN). **Middle:** The same culture field was examined immediately POST-scratch injury (IMM). The diagonal (from top left to bottom right) region where the plastic gel-loading pipette tip was used to injure myoblasts. The white rectangle highlights a mass of cellular debris within the region of injury. **Right:** The same field of myoblasts shown in left and middle images, taken at 24h post injury. By 24h post-injury, the cleared region shown IMM POST is completely filled in with myoblasts. The white rectangle is the same location shown in the IMM POST image. Images were taken at 10X magnification.
Figure 4.5. Images of uninjured control myotube cultures at baseline (BSLN) through 48h (48H). **Top:** The BSLN image at top left is not the same as the rest of the time points, but it is representative of uninjured controls. The remaining images are of the same field of uninjured controls taken immediately post-injury and 1, 3, 6, 24, and 48h post-scrape injury with a plastic scraper. Myotubes undergo relatively minor changes in gross morphology from BSLN to 48h (time series shown from left to right, top to bottom). The cells in these images were in the same 10cm dish as the injured cells shown below in Figure 4.6. Images were taken at 10X magnification.

Figure 4.6. Images of injured myotube cultures at baseline (BSLN) through 48h (48H). **Top:** The BSLN image at top left is not the same as the rest of the time points but it is representative of uninjured controls. The plastic scraper induces a clean injury region that was followed at the time points indicated in each image. **Left to right:** The vertical myotube in the top right corner of each image is transected IMM POST, undergoes repair events at 1, 3, and 6h, and then appears to fully regenerate and increase in size by 24 and 48h post injury. Other post-injury myotube repair events are also evident in this region of injury, as well as other fields in the culture dish, as shown below in Figure 4.7. Images were taken at 10X magnification.
Figure 4.7. Images of injured myotube cultures at 6–48h (6H–48H) post injury. The images at right are the same field of injured cells at 6, 24, and 48 h, taken at 10X magnification (left column), and 20X magnification (right column). Here, myoblasts migrate into the region of injury within ~6 h (Top row). By 24h, migration is increased, relative to 6h, and proliferation also appears to occur (Middle row). At 48 h post, migration and proliferation appears to continue as myoblasts form a new monolayer within the region of injury (Bottom row).
Myotube cultures under normal and injured conditions display typical morphologies during an extended 72h protocol

Figure 4.8. demonstrates that normal and injured myotube cultures display similar morphologies, relative to all previous time points (i.e. BSLN through 48h), for the duration of the 72h time-series, and illustrates the extent of muscle regeneration over 72h following scratch-injury. The typical time course for the C₂C₁₂ myotube culture experiments shown in Figures 4.4.-4.7 was up to 48h. To ensure the feasibility of conducting experiments over an extended 72h time-series, additional pilot experiments were conducted using the identical experimental design and methods as described above. The results of those experiments are illustrated below in Figures 4.8.-4.10. It is noteworthy to mention that the only differences between the 48h and 72h experiments were the addition of the 72h time point (Figures 4.8.-4.10.), as well as the addition of the PR39 treatment condition (Figures 4.9.-4.10. only).

Figure 4.8. Scratch injury induces satellite cell migration and regeneration of injured myotube cultures. Representative images of untreated uninjured control (top panels) and untreated scratch-injured (bottom panels) myotube cultures at baseline (BSLN), 24 hours (24H), 48 hours (48H), and 72 hours (72H) post-injury. Images were taken at 10X magnification.
**PR39 dose and time-response pilot experiments**

Control and injured myotube cultures were incubated with 1, 5, or 10μm PR39 or an equivalent volume of PBS vehicle control. Myotube cultures were treated with PR39 1h prior to the initiation of each experiment and images of control and injured cultures were collected at baseline and 24, 48, and 72h post-injury. Muscle regeneration was evaluated at the anatomical level using phase contrast microscopy at 10X magnification, as illustrated below in Figures 4.9-4.10.

**PR39 at concentrations >1μm are lethal to myotubes but not satellite cells under otherwise normal conditions**

Figure 4.9 demonstrates that uninjured control myotube cultures treated with 1μm PR39 at 1h prior to BSLN display similar morphologies to untreated controls over 73h (BSLN-72h) post-treatment. However, 5μm and 10μm dosages of PR39 have toxic effects on myotubes but do not appear to negatively affect satellite cell morphology or number.
**Figure 4.9.** Dose and time effects of 1, 5, and 10µM PR39 on control myotube cultures. Representative images of untreated controls (top row), cultures treated with 1µM PR39 (2nd row from top), cultures treated with 5µM PR39 (3rd row from top) and cultures treated with 10µM PR39 (bottom row) at 1h prior to baseline (BSLN), 24 hours (24H), 48 hours (48H), and 72 hours (72H). Scratch-injured (bottom panels), 24 hours (24H), 48 hours (48H), and 72 hours (72H) post-injury. Dose-dependent toxicity is apparent by viewing the images within each column from top to bottom and the time-dependent toxicity of PR39 is apparent by viewing each row from left to right. Images were taken at 10X magnification.

**PR39 at concentrations >1µm are lethal to myotubes but not satellite cells during regeneration**

This figure (figure 4.10.) demonstrates that injured myotube cultures treated with 1µm PR39 at 1h prior to BSLN display similar morphologies to untreated injured cultures over 73h (Imm post-72h) post-treatment. However, 5µm and 10µm dosages of PR39 have toxic effects on myotubes but do not appear to negatively affect satellite cell morphology.
or number. Interestingly, satellite cells at all time points, independent of PR39 concentration, exhibit similar morphologies, migration rates into wound regions and proliferation rates (evidenced by the apparent increase in mononucleated cell densities in wound regions) at 24-72h post-injury.

Figure 4.10. Dose and time effects of 1, 5, and 10μM PR39 on injured myotube cultures. Representative images of untreated injured cultures (top row), injured cultures treated with 1μm PR39 (2nd row from top), injured cultures treated with 5μm PR39 (3rd row from top) and injured cultures treated with 10μM PR39 (bottom row) taken immediately-post injury (Imm-post), and at 24 hours (24H), 48 hours (48H), and 72 hours (72H). Dose-dependent toxicity (phase bright rounded cells) is apparent by viewing the images within each column from top to bottom and the time-dependent toxicity of PR39 is apparent by viewing each row from left to right. Images were taken at 10X magnification.
Conclusions

The gel-loading pipette tip method induced mechanical injury to undifferentiated cultures of myoblasts. Myoblasts were observed migrating into the regions of injury within ~3h. Complete wound closure, indicated by the similar appearance of the field of injured myoblasts at 24h post, compared to BSLN, was observed within ~24h, which was similar to the time course (i.e. within 24h) observed in the study by Yahaoui et al. (115). Experiments performed in heterogeneous cultures of satellite cells and myotubes (i.e. myotube cultures) resulted in migration and proliferation of satellite cells to the injured regions that was similar to the time course (i.e. within 24 h) observed in the undifferentiated cultures of myoblasts scratch-injury experiment shown in Figure 4.4. Regeneration and growth of the injured myotubes within the cultures was evident ~24-48h. These data demonstrate a similar time course (i.e. within ~24-48h) of satellite cell proliferation and differentiation to observations made in previous studies (101, 115) using the same model.

It was determined from the experiments illustrated in Figures 4.9.-4.10. that 1μm PR39, delivered in a single bolus at 1h prior to the execution of scratch-injury, was the maximal concentration that could be used without inducing visually detrimental effects on differentiated myotubes within the heterogeneous cultures of satellite cells and myotubes used in this work. The 5-10μM dosages of PR39 induced an apparent degeneration of the myotubes within the cultures, as indicated by the dramatic decrease in myotube size and number, yet did not appear to negatively affect the satellite cells. It is unclear from the anatomical biology of those experiments, observed through phase-contrast microscopy, what the precise mechanisms are for selective toxicity toward myotubes and not satellite cells (in normal controls) or activated satellite cells during
regeneration. However, toxic effects were not observed in cultures treated with 1\(\mu\)m PR39. Therefore, to determine the effects of PR39 on NF-kB transactivation and MCP-1 protein secretion following scratch-injury, myotube cultures were treated with a final concentration of 1\(\mu\)M PR39 at 1h prior to each experiment in Study II (Chapter V) of this dissertation. In addition, untreated vehicle controls received an equivalent volume of PBS (PR39 vehicle) at 1h prior to each experiment, which is a common reagent used to rinse cells during standard C\(_2\)C\(_{12}\) subculturing procedures. Therefore, the experimental design and methods used to develop this in vitro model of muscle injury were used to test the hypotheses for Study II in the following chapter.
CHAPTER V
NF-KB ACTIVATION AND MCP-1 SIGNALING FOLLOWING ACUTE MUSCLE INJURY

Introduction

Acute skeletal muscle injury is a common clinical condition caused by unaccustomed physical exertion, blunt or lacerative trauma, ischemia, and/or exposure to toxic chemicals. Skeletal muscle has an innate capacity for self-repair following acute injury, yet the underlying processes are complex and poorly understood. Acute skeletal muscle injury is followed by both muscle degeneration and subsequent regeneration leading to the restoration of muscle function. Post-injury regeneration is broadly characterized by the focal activation and proliferation of the quiescent muscle stem cells, known as satellite cells, which migrate to the site of injury where they fuse with injured muscle fibers (87, 110). Inflammation (89, 90) is an essential regulator at each step of these processes.

Muscle injury results in a well-characterized inflammatory response involving the recruitment of immune cells, which play critical roles in both the degenerative and regenerative phases of repair, including the activation, proliferation, and fusion of satellite cells with multinucleated myofibers (3, 22, 36). The acute or degenerative phase is characterized by the secretion of pro-inflammatory molecules from the injured tissue, which recruit circulating leukocytes, such as monocytes and macrophages, to the site of injury (4, 20, 102). Leukocytes secrete pro-inflammatory effectors that activate satellite cells, degrade necrotic myofibers by releasing myotoxins (i.e. reactive oxygen and nitrogen species), and by phagocytosing cellular debris (8, 96, 102, 103).
However, the degenerative inflammatory processes can induce secondary damage to the injured tissue, and prolong repair and regenerative processes during the subsequent anti-inflammatory phase. These processes are characterized by the activation of anti-inflammatory macrophages, restoration of muscle strength and resolution of inflammation (76, 93, 94, 102, 103). Although inflammation appears to regulate both the biochemical signaling between injured skeletal muscle and the immune system, and the physical remodeling and regeneration of skeletal muscle after acute injury, the molecular mechanisms that mediate these responses remains largely unknown. Determining how muscle inflammation regulates muscle repair is essential for developing strategies to reduce post-exercise/traumatic muscle injury and promote subsequent recovery.

Recent in vivo and in vitro studies have implicated both nuclear factor-kappa beta (NF-κB) and monocyte chemoattractant protein-1 (MCP-1) as important inflammation factors that regulate the communication between muscle cells, monocytes, and macrophages following acute skeletal muscle injury (3, 5, 9, 36, 38, 111). NF-κB belongs to a family of transcription factors that regulate the expression of over 150 genes, including pro-inflammatory mediators of muscle repair processes and macrophage function (42, 57). In skeletal muscle, NF-κB has been shown in in vitro and in vivo models to regulate myogenesis and protein degradation, as well as induce pro-inflammatory mediators such as MCP-1 and iNOS in response to acute injury (5, 42, 51, 100). MCP-1, also known as CC chemokine ligand 2 (CCL2), is a ligand of C-C chemokine receptor 2 (CCR2), which recruits circulating monocytes and macrophages to damaged muscle tissue and activates resident macrophages (3, 9).
The first evidence that MCP-1 expression was dramatically elevated after traumatic injury was provided by Warren et al (112), using an acute freeze-injury model in rodents. They observed a ~100 fold increase in MCP-1 mRNA expression at 24h post-injury relative to controls. Using an eccentrically-biased, exercise-induced muscle damage (EIMD) model in humans, Hubal et al. (36) reported a 9.2 fold increase in MCP-1 mRNA and co-localization of MCP-1 expression in macrophages and satellite cells in damaged muscle tissue ~6h after a bout of damaging exercise of the quadriceps. Furthermore, Hylldahl and colleagues (38) found a 17.9 fold increase in MCP-1 mRNA and 1.6 fold upregulation of NF-kB DNA binding activity in human muscle biopsies at 3h post-exercise. Using a similar EIMD model, Vella and colleagues (107) demonstrated NF-kB nuclear transactivation and DNA binding of the MCP-1 gene promoter (via *electrophoretic mobility shift assay (EMSA)*), which suggests that NF-kB plays a role in upregulating MCP-1 mRNA expression following acute EIMD.

These and other studies demonstrate that NF-κB activity and MCP-1 transcript abundance are rapidly upregulated in damaged muscle tissue. While molecular studies have shown that MCP-1 expression can be influenced by NF-κB expression, it is still not known if these two processes are independently activated by injury or if NF-κB activity is induced by injury and, in turn, drives MCP-1 expression. It is also unclear if NFkB activation and MCP-1 up-regulation are critical to normal muscle regeneration and wound healing. Therefore, the purposes of this study were to: 1) determine the relationship between NF-κB activation and MCP-1 expression in muscle cells in response to an acute lacerative injury; and 2) determine the roles for each in post-injury repair.
We utilized an acute lacerative-injury model in C\textsubscript{2}C\textsubscript{12} myotube cultures to determine the early temporal responses of and relationship between NF-κB and MCP-1 following injury. We hypothesized that muscle cell injury would induce an increase in NF-κB transactivation leading to increased cytosolic MCP-1 protein expression and secretion into the culture medium. We tested this interdependence via blockade of NF-kB transactivation, which we hypothesized would decrease MCP-1 expression and secretion following injury, relative to injured controls. We also hypothesized that changes in NF-kB activation and MCP-1 would correlate to repair indices, which would be attenuated by blockade of NF-kB transactivation.

**Methods**

**Myogenic Cell Culture**

C\textsubscript{2}C\textsubscript{12} myoblasts [(a mouse satellite cell line; Yaffe and Saxel, 1977 (99)] purchased from *American Type Culture Collection* (ATCC, Manassas, VA) were cultured in 10cm\textsuperscript{2} sterile polystyrene tissue culture dishes in the presence of growth medium (GM) composed of: Dulbecco’s Modified Eagle Medium (DMEM) (GIBCO, Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Atlanta, GA) and 1% penicillin/streptomycin (GIBCO, Invitrogen Corporation, Carlsbad, CA) at 37°C in the presence of 10% CO\textsubscript{2}. Cultures were fed fresh GM every 24h. When cultures reached \textasciitilde80% confluency, the medium was aspirated and the cells were rinsed once with 1 X phosphate buffered saline (PBS), before incubation with 3ml 0.5% trypsin-EDTA (GIBCO, Invitrogen Corporation, Carlsbad, CA) for 4 minutes (min) at 37°C to lift cells from the bottom of the culture dish prior to transferring them to sterile 15ml conical polystyrene tubes (Fisher Scientific, USA) containing 3ml fresh DM to halt the trypsinization process.
Cells were then pelleted by centrifugation at 4000 x g for 90 seconds (s). The supernatant was then aspirated and the cell pellet was resuspended in 2ml fresh GM via 40 cycles of repeat pipetting with a rubber bulb and glass Pasteur pipette. A 40μL aliquot of cell suspension was then diluted into 40μL Trypan Blue dye (GIBCO, Invitrogen Corporation, Carlsbad, CA) and incubated at room temperature for 2 min. Then, 10μL of the Trypan Blue-containing cell suspension was applied to each chamber of a Neubauer hemacytometer and cell viability was determined under a Nikon TMS inverted microscope via the Trypan Blue exclusion method per the manufacturer’s protocol. Cells that excluded Trypan Blue dye were deemed to be alive while those that stained blue were counted as dead and were excluded from the total viable cell count. The cell suspension was then serially diluted in GM and seeded at a standardized density in sterile tissue culture dishes to be used for experiments. Cells were monitored and GM was aspirated and replaced with fresh GM every 24h until the cells reached ~80% confluency. GM was then aspirated and replaced with differentiation medium (DM) composed of: DMEM supplemented with 2% horse serum and 1% penicillin/streptomycin (GIBCO, Invitrogen Corporation, Carlsbad, CA) at 37°C in the presence of 10% CO2. Cultures were then fed fresh DM every 24h until myoblast cultures differentiated into mature, multi-nucleated myotubes. Myoblasts were allowed to differentiate into myotubes for 120h prior to experimentation. During the differentiation period, DM was then aspirated and replaced with fresh DM every 24h.

**In Vitro Scratch-Induced Muscle Injury Model**

C2C12 myotube cultures were differentiated for 5d and then subjected to scratch-injury to create an open “wound” on the culture plate. On the morning of each experiment, DM was replaced with fresh DM and cultures were incubated for 3h to allow
the cells to equilibrate to fresh DM. Cultures were then scratch-injured with a sterile gel-loading pipette tip to generate a continuous and well-delineated wound region along the surface of each plate, which produced a total wound area equivalent to ~10% of the total surface area of each plate. Samples of DM, as well as cytosolic and nuclear extracts, were collected from uninjured controls and injured myotube cultures at baseline, 1, 3, 6, 12, 19, 24, and 72h post-injury and immediately frozen at -80°C for ELISA analyses of NF-kB transactivation and MCP-1 protein expression. Phase-contrast and fluorescence images were collected from uninjured controls and injured cultures at the aforementioned time points with a Nikon model TMS inverted microscope and a SPOT Insight imaging color camera (Diagnostic Instruments, Sterling Heights, MI) to evaluate regeneration of injured myotube cultures pre- to post-injury. In addition, the aforementioned experiments were conducted in the presence or absence of PR39, which is a specific inhibitor of NF-kB/p65 subunit transactivation (27). The experimental design and measures are shown in Figure 5.1.
**Figure 5.1.** Experimental Design for *In Vitro* Muscle Injury. All experiments were performed independently and 3-6 biological replicates were collected per time point and under each condition (control or injured in presence/absence of 1µm PR39). Measures were collected at baseline and 1, 3, 6, 12, 19*, 24, and 72h* post; “*” indicates satellite cell proliferation & fusion assay time points (described below). Qualitative Measures: Phase-contrast and fluorescence images of cultures labeled for NF-κB/p65 localization using immunocytochemistry and fluorescence microscopy (ICC & FM). Quantitative Measures: NF-κB/p65 DNA Binding activity in nuclear extracts (ELISA), MCP-1 protein expression in cytosolic extracts and DM (ELISA); Satellite cell proliferation & fusion assays (ICC & FM).

**Pharmacological blockade of NF-κB transactivation**

PR39 (R&D Systems, Minneapolis, MN, Catalog #1947) is a porcine-derived peptide inhibitor of NF-κB transactivation. PR39 selectively (and reversibly) binds the α7 subunit of the 20S proteosome and blocks Iκβα (the cytosolic inhibitor of the NF-κB p65/p50 heterodimer) degradation by the ubiquitin proteasome pathway without disrupting overall proteasome activity (27). With this in mind, PR39 was chosen for this research because it has been shown *in vitro* in various cell lines to suppress classical NF-κB pathway-dependent gene expression both in cell culture and in two models of acute injury in mice (27).

Dose-response experiments were conducted at the outset of this dissertation study to empirically determine the optimal concentration of PR39, and the treatment-duration...
of myotube cultures, as described in detail in Chapter IV. From those experiments, it was determined that 1μm PR39, delivered in a single bolus at 1h prior to the onset of scratch-injury, was the maximal concentration that could be used without inducing detrimental effects (i.e. complete disappearance) of differentiated myotubes within the heterogeneous cultures of satellite cells and myotubes used in this work. Therefore, to determine the effects of PR39 on NF-κB transactivation and MCP-1 protein secretion following scratch-injury, myotube cultures were treated with a final concentration of 1μM PR39 at 1h prior to each experiment. Untreated vehicle controls received an equivalent volume of PBS (PR39 vehicle) at 1h prior to each experiment.

**Immunofluorescence detection of NF-κB transactivation in C₂C₁₂ myotube cultures**

At baseline, 12, and 24h, control and injured myotube cultures, grown on Nunc (ThermoScientific, Pittsburg, PA) slide flasks, were fixed for 2 min in 2% paraformaldehyde in PBS at room temperature. The cultures were then permeabilized for 3 min with Karsenti’s Lysis Buffer (0.5% Triton X-100, 80 mM PIPES, 1.0 mM MgSO₄, 5.0 mM EGTA, pH 7.0), rinsed twice with PBST (PBS, 0.1% Tween-20), and blocked with 2% albumin in PBST for 15 min. Then, cultures were incubated with primary rabbit α-mouse monoclonal antibody (1:200; Abcam, Catalog #7970-1) against NF-κB/p65 in PBST for 1h in darkness at room temperature. After 3 washes with PBST, the cells were incubated with a secondary antibody (1:1000; AlexaFluor 488, goat α-rabbit (Life Technologies, Grand Island, NY) for 60 min in darkness at room temperature. Myotube cultures were washed again 3 times with PBST and then incubated with the nuclear label 4,6-diamidino-2-phenylindole (DAPI) (1:100; Sigma-Aldrich, St. Louis, MO) for 15
min in darkness at room temperature. After 3 washes with PBST and one wash with PBS, slides were coverslipped and visualized with a Nikon model TMS inverted microscope and a SPOT Insight imaging color camera (Diagnostic Instruments, Sterling Heights, MI).

Quantification of NF-κB/p65 subunit transactivation

Nuclear extracts were isolated from C_{2}C_{12} myotube cultures via differential centrifugation using a cellular extraction kit (Nuclear Extract Kit™, Active Motif; Catalog #40010) and immediately frozen at -80 °C. Total nuclear protein content was then determined using a BSA-based protein quantification assay (ProStain™, Active Motif; Catalog #15001). An ELISA-based transcription factor assay kit (TransAM™ NF-κB Family Transcription Factor Assay Kit, Active Motif; Catalog #40096) was used to detect and quantify NF-κB/p65 subunit nuclear DNA binding activity in uninjured controls and injured myotube cultures, in the presence or absence of PR39 at each time point. All assays for sub-cellular extractions, protein quantification, and NF-kB/p65 subunit transactivation were performed according to the manufacturer’s protocols.

To quantify p65 transactivation, 30μl of complete Binding Buffer AM3 was first added to each well on the 96-well plate. 20μl of C_{2}C_{12} nuclear extracts diluted to 1μg/μl protein in Complete Lysis Buffer AM2 (CLB), containing 5μl of 1M DTT and 10μl of Protease Inhibitor cocktail per ml CLB, as well as negative controls (blanks, 20μl CLB only), were then added to each well and technical replicates were run in duplicate on each plate. The plate was then sealed and incubated on a rocking platform (100 rpm) for 1h at room temperature. After incubation, the plate was washed 3 times with 200μl Wash
Buffer AM2 per well. During each wash cycle, the plate was also carefully blotted 4 times on a fresh stack of 10 Kim Wipes, rotated 180°, and blotted again 4 times on a fresh stack of 10 Kim Wipes to remove all Wash Buffer AM2 from each well and avoid cross-contaminating wells on the plate with discarded ELISA reagents on used Kim Wipes. 100µl of NF-κB/p65 subunit primary antibody solution (1:1,000 in Antibody Binding Buffer AM2) was then added to each well, and the plate was again sealed and incubated for 1 h at room temperature. Following incubation, the plate was washed 3 times, and then 100µl of HRP-conjugated secondary antibody solution (1:1,000 in Antibody Binding Buffer AM2) was added to each well. The plate was again covered and incubated for 1 h at room temperature. Following incubation, the plate was washed 4 times with 200µl of Wash Buffer AM2 per well. 100µl of Developing Solution was then added to each well and then the plate was incubated in the dark for 5 min at room temperature. 100µl of Stop Solution was then added, and the plate was read using an A450 absorbance filter on a microplate reader (FLUOstar Optima, BMG Labtech INC., USA) at 450nm. The raw data [(optical density values, (OD450)] were then normalized by subtracting the blank values from all other values.

**Quantification of MCP-1 Protein Expression**

Cytosolic extracts were isolated from the exact same C2C12 myotube cultures that NF-κB transactivation was quantified from via differential centrifugation using a cellular extraction kit (Nuclear Extract Kit™, Active Motif, Catalog #40010) and immediately frozen at -80°C. Total protein content in cytosolic extracts and culture medium were then determined using a BSA-based protein quantification assay (ProStain™, Active Motif,
Catalog #15001) in uninjured controls and injured myotube cultures, in the presence or absence of PR39 at each time point. All assays for sub-cellular extractions and protein quantification were performed according to the manufacturer’s protocols.

A colorimetric ELISA assay kit (Quantikine® Mouse CCL2/JE/MCP-1 Immunoassay, R&D Systems, Minneapolis, MN; Catalog #MJE00) was then used to quantify cytosolic MCP-1 protein expression in myotube cultures, as well as MCP-1 protein concentrations in culture media samples collected from the exact same wells that cell extracts were collected from. All procedures for the assay were performed according to the manufacturer’s instructions. The assay kit employs the quantitative sandwich ELISA technique and 96-well plate format. Each 96 well plate is pre-coated with an affinity purified polyclonal antibody specific for mouse MCP-1.

Samples of the culture media from injured myotube cultures were assessed undiluted, whereas cytosolic extracts from myotube cultures were diluted (1:5) in ELISA Assay Diluent RD1W. 50μl of Assay Diluent RD1W was added to each well of the provided 96 well plate. 50μl of Mouse/JE/MCP-1 (Standard), Recombinant Mouse/JE/MCP-1 Control (positive control), negative control (i.e. Assay Diluent RD1W only), or samples from C2C12 cytosolic extracts or culture media were added per well and then gently mixed on a gyratory shaker for 1 min. All standards, controls and C2C12 samples at each time point and condition were assayed in duplicate (i.e. 2 technical replicates for each biological replicate). The plate was then covered with an adhesive strip and incubated at room temperature for 2h. Each well was then aspirated and washed with 400μl of Wash Buffer for a total of 5 aspiration/wash cycles. During each wash cycle, the plate was also carefully blotted 4 times on a fresh stack of 10 Kim Wipes,
rotated 180 degrees, and blotted again 4 times on a fresh stack of 10 Kim Wipes to
remove all Wash Buffer from each well and avoid cross-contaminating wells on the plate
with discarded ELISA reagents on used Kim Wipes. 100µl of Mouse/JE/MCP-1
Conjugate was then added to each well and the plate was then covered with a new
adhesive strip and incubated for 2h at room temperature.

At the end of the /JE/MCP-1 Conjugate incubation period, the identical
aspiration/wash/blotting cycles were repeated. Then, 100 µl of Substrate Solution,
containing a 1:1 mixture of Color Reagents A and B, was added to each well. The plate
was again sealed and incubated for 30 min at room temperature in darkness. Next, 100 µl
of Stop Solution was added to each well and thoroughly mixed by placing the plate on a
gyratory shaker for 1 min.

The optical density of each well was then determined immediately on a
microplate reader (FLUOstar Optima, BMG Labtech INC., USA) at 450nm with a
correction wavelength of 540nm. The raw data (corrected optical density values) were
then normalized by subtracting the blank values from the standard, control, and C2C12
sample values. A standard curve was created, using the instrument software (MARS
system software, BMG Labtech Inc., USA), by reducing the data with a four parameter
logistic (4-PL) curve-fit, as recommended by the manufacturer’s instructions. MCP-1
concentrations in C2C12 cytosolic extracts and culture media samples were then plotted
against the standard curve created from the purified recombinant MCP-1 standard. The
concentrations of diluted samples (i.e. cytosolic extracts from myotube cultures
containing satellite cell and myotube lysates) read from the standard curve were then
multiplied by the dilution factor to obtain the accurate predicted MCP-1 concentrations from C2C12 cytosolic extracts and culture media samples.

**Quantification of satellite cell proliferation and fusion**

A 5-Bromo-2’-deoxy-uridine (BrdU) Labeling and Detection Kit (Roche Applied Science, Indianapolis, IN) and fluorescence microscopy were used to determine if scratch-injury induced satellite cell proliferation and subsequent fusion of those satellite cells with the differentiated myotubes in the control and injured conditions at 19h and 72h post. Myotube cultures grown in 6-well culture dishes were induced to differentiate for 5d prior to experiments and 3 independent experiments were performed in order to collect 3 biological replicates per condition, per time point.

On the morning of each experiment, culture medium was replaced with fresh DM and cultures were incubated for 3h to allow the cells to equilibrate with fresh DM. Then, immediately prior to each experiment, BrdU was added to cultures under each condition at a final concentration of 10μM (per manufacturer’s instructions) and incubated for 19h (N=3) or 72h (N=3) after the onset of the experiment. The 19h and 72h time points were chosen based on evidence that satellite cells undergo one complete round of proliferation within ~18h and myogenic fusion of satellite cells with differentiated myotubes can occur within 72h following injury. At each time point, culture medium containing BrdU was aspirated and cells were rinsed 3 times with 700μl Wash Buffer per well and then fixed in ethanol-acid fixative at -20°C for 25 min. Then, cells were rinsed 3 times with Wash Buffer and incubated with an α-BrdU monoclonal antibody, diluted 1:10 in α-BrdU Working Solution for 30 min at 37°C. Cultures were then rinsed 3 times with Wash
Buffer and incubated with AlexaFluor 594 α-mouse secondary antibody diluted 1:400 in Secondary Antibody Solution for 30 min at 37°C. Thereafter, cultures were rinsed 3 times with Wash Buffer and incubated in 49,6-diamidino-2-phenylindole (DAPI) (Sigma, USA) diluted 1:100 in PBS for 30 min at room temperature and again rinsed 3 times with PBS. Cultures were then briefly air-dried and then each well was coverslipped using mounting medium containing 1% propylgallate in glycerol and 4mm x 4mm cover glass in each well, and sealed with clear nail polish. 5 random images per replicate of each condition were collected on a Nikon model TE-2000 inverted microscope and a SPOT Insight imaging color camera (Diagnostic Instruments, Sterling Heights, MI) at 10x magnification.

Satellite cell proliferation was quantified by counting the total number of BrdU positive (BrdU⁺) nuclei and DAPI positive (DAPI⁺) nuclei within each image field using the ImageJ-ITCN plug-in cell counting function (ImageJ, www.NIH.gov). An index of cell proliferation was determined by the calculating the ratio of BrdU⁺: DAPI⁺ nuclei within each image field.

Satellite cell fusion was quantified from the same images by manually scoring the number of BrdU⁺ nuclei within each myotube boundary. Then, the BrdU⁺ images were merged with the corresponding phase-contrast image to verify the incorporation of BrdU⁺ nuclei into differentiated myotubes. In order for a satellite cell to be confirmed as incorporated into a myotube, the entire BrdU⁺ nucleus had to reside entirely within the boundaries of the myotube membrane.
Statistical Analysis

Using the Statistical Analysis Software program (SAS Institute, Cary, NC, USA), a two-way ANOVA with main effects of “Treatment” and “Time” and the “Treatment by Time” interaction were calculated from ELISA data. The results were tested for normality and equal variance and are reported as means ± SEM of at least 3-6 biological replicates per condition. When significant effects were detected, a Tukey’s honest significant difference test (post-hoc) was used to determine differences between means. Cross sectional analyses via Student’s unpaired t-tests were also used to reveal significant treatment effects at specific time points of interest along the complete time series. In addition, Pearson Product Moment Correlations were used to determine if changes in NF-kB transactivation were significantly correlated with changes in MCP-1 expression. Significance was set a priori at p<0.05.

Results

NF-kB/p65 cellular localization in myotube cultures

To further explore the source of NF-kB/p65 activation among both satellite cells and myotubes within C2C12 cultures, the cellular localization of the p65 subunit was examined in uninjured controls and injured myotube cultures at 12h post-injury via immunocytochemistry and fluorescence microscopy (Figure 5.2.). In control cultures (under normal conditions), NF-kB/p65 protein appeared to be highly abundant in the cytoplasm of both multinucleated myotubes and in mononucleated satellite cells, with some apparent concentration in the perinuclear region of the satellite cells. I observed p65 protein colocalized to satellite cell nuclei and myotube nuclei (closed arrows) in both
control (left panels) and injured cultures (right panels) at 12h, and at the anatomical level, there appeared to be greater nuclear p65 accumulation in satellite cells compared with myotubes. Notably, myotubes in these C2C12 cultures have a high-density of nuclei, which are randomly distributed throughout the cell, as illustrated by the blue nuclear staining (DAPI, bottom panels) within the myotube borders.

Myotubes in culture also exhibit random morphologies, such that some exist as single tube-like structures and others appear to have branch-like morphologies resembling two or more single myotubes fused together. Furthermore, myotubes have considerably greater mass and volume than do satellite cells, and some of their myonuclei reside toward the inferior surface (closest to the plate surface) of the cell, whereas others are clearly visible toward the superior surface of the myotube. It is plausible that some of the myotube nuclei that are localized to the inferior surface of the cell, and appear to contain p65, may not actually contain p65. Rather, they reside underneath a non-nuclear region of the myotube that does contain p65, and higher resolution of the nuclear compartments may be better achieved using confocal microscopy as opposed to epifluorescence microscopy used in these images.

Conversely, I observed several examples of myonuclei devoid of a p65-specific signal (open arrows), which were also apparent within both control and injured cultures, as evidenced by the absence of green fluorescence within the nuclear border of both satellite cells and myotubes. Those observations suggest that in p65 protein was absent from those particular myonuclei, under normal conditions (controls) and in regenerating myotube cultures (injured).
Taken together, in these heterogeneous C₂C₁₂ myotube cultures, myonuclear accumulation of p65 appeared greater in satellite cells than in myotubes in both the control and injured cultures at 12h, which suggests that activated p65 in satellite cells may be a primary source of the net p65 signal from both cell types (satellite cells and myotubes) in my model. Further, those data suggest that p65 remains constitutively active in satellite cells in these cultures, which corroborates previous findings in C₂C₁₂ models of myogenesis (5, 109). However, in the present study, this cannot be concluded solely from the qualitative observations made at the anatomical level using ICC and FM. Therefore, I took a more sensitive and quantitative biochemical approach to further explore these findings using isolated nuclear extracts derived directly from control and injured myotube cultures. The biochemical assay data presented in Figure 5.2., offer a more accurate depiction of net p65 transactivation in myotube cultures following scratch-injury.
**Figure 5.2.** Cellular localization of NF-kB p65 subunit following scratch injury. Two representative images at 20X magnification of myotube cultures immunolabeled with an antibody against the p65 protein subunit of NF-kB (green, top and bottom panels) and the myonuclei counterstained with DAPI to visualize nuclei (blue, bottom panels) in uninjured control (left panels) and injured (right panels) cultures at 12h post-injury. Open arrows indicate nuclei that appear to be void of p65 protein. Closed arrows indicate p65 protein localized to nuclei of mononucleated satellite cells and in multinucleated myotubes in control and injured conditions. Images indicate that the NF-kB/p65 subunit is a highly abundant protein in C2C12 muscle cells.

**NF-kB/p65 subunit transactivation decreases following scratch-injury**

To further characterize nuclear p65 activation in normal myotube cultures, and to test the hypothesis that scratch-injury would increase p65 transactivation within 24h, p65 subunit DNA binding activity was measured in C2C12 nuclear extracts at baseline (BSLN) and at 1, 3, 6, 12, and 24h post-injury via an NF-kB/p65 DNA binding ELISA assay (Figure 5.3.). A significant main effect for time (p<0.01), and treatment x time interaction
(p<0.01) was observed, with no main effect of treatment (p=0.14) in myotube cultures pre- to post-injury. Normal control cultures exhibited a variable temporal pattern of p65 DNA binding activity, which increased by 313±17% over 24h and was significantly (p<0.04) different from BSLN by 1h. Those data indicate that p65 is constitutively active in myotube cultures and transiently increases over 24h under normal conditions. Injured cultures displayed a similar temporal pattern of p65 activation, which increased by 333±26% over 24h relative to BSLN (p<0.001). In comparison to normal cultures, p65 activation was significantly (p<0.02) decreased by 37±4% at 6-12h, indicating that p65 transactivation is decreased in response to scratch-injury.

**Cytosolic MCP-1 protein expression increases in injured myotube cultures following scratch-injury**

To determine the levels of cytosolic MCP-1 protein in normal myotube cultures and to test the hypothesis that scratch-injury would induce cytosolic MCP-1 expression within 24h, ELISA assays were performed to determine MCP-1 protein concentrations in C2C12 cytosolic extracts derived from control and injured myotube cultures at BSLN and at 1, 3, 6, 12, and 24h (Figure 5.4). A significant main effect for time (p<0.039), and treatment (p<0.001) was observed, with no treatment x time interaction (p=0.09) in myotube cultures pre- to post-injury. In control cultures, the levels of cytosolic MCP-1 declined 44±10% over 24h and were significantly (p<0.04) different from BSLN by 1h, indicating that under normal conditions, intracellular MCP-1 levels decreased within 1h and remained relatively constant until 24h. In contrast, injured cultures displayed the opposite pattern of expression and accumulated (36±19%) cytosolic MCP-1 protein at 3h
post-injury (relative to BSLN), which tended toward statistical significance (p=0.07). Compared to uninjured controls, injured cultures displayed a 96±24% increase in cytosolic MCP-1 protein over the first 6h, which was significantly (p<0.02) different from uninjured controls by 1h post-injury.

**Figure 5.3.** Effects of scratch injury on NF-kB/p65 transactivation pre- vs. post-injury. Data shown as optical densitometry values (OD450) for p65 DNA binding activity in C2C12 nuclear extracts at baseline (BSLN) and 1-24 hours (H) in uninjured control (open bars, N=3) and injured (closed bars, N=3) cultures, M ± SEM. Asterisk (*) with solid line indicates significantly different from BSLN (p<.05); †significantly different from uninjured control at each time point (p<.05).
**Figure 5.4.** Cytosolic MCP-1 protein expression in control and scratch-injured myotube cultures. Data are shown as absolute cytosolic MCP-1 protein concentrations, expressed relative to total cytosolic protein concentrations (pg MCP-1 µg⁻¹ total cytosolic protein) in C₂C₁₂ cytosolic extracts at baseline (BSLN) and 1-24 hours (H) in uninjured control (open bars, N=3) and injured (closed bars, N=3) cultures, M ± SEM. *Significantly different from BSLN (p<0.05); †significantly different from uninjured control at each time point (p<0.05).

MCP-1 protein secretion increases from control and injured myotube cultures following scratch-injury

To characterize MCP-1 protein secretion from normal myotube cultures and to test the hypothesis that scratch-injury would induce a significant increase in MCP-1 protein secretion, ELISA assays were used to quantify MCP-1 protein in C₂C₁₂ DM at BSLN and at 1, 3, 6, 12, and 24h in control and injured myotube cultures (Figure 5.5.). A significant main effect for time (p<0.001), treatment (p<0.001) and treatment x time interaction (p<0.004) was observed in myotube cultures pre- to post-injury. In normal control cultures, MCP-1 secretion increased by 576±10% over 24h and was significantly (p<0.001) different from baseline by 12h, indicating a diurnal increase in MCP-1
secretion from myotube cultures under normal conditions. Injured cultures displayed a similar pattern of MCP-1 secretion, which increased by 820±43% over 24h and was significantly (p<0.001) different from BSLN by 12h post-injury. Compared to uninjured controls, injured cultures displayed a 93±37% increase in MCP-1 secretion at 12-24h, which was significantly (p<0.02) different from uninjured controls by 12h post-injury, indicating that scratch-injury induced MCP-1 secretion from myotube cultures.

**Figure 5.5.** MCP-1 protein secretion from uninjured control and injured myotube cultures into culture medium as determined by ELISA. Data are shown as absolute MCP-1 protein concentrations in C2C12 culture medium at baseline (BSLN) and 1-24 hours (H) in uninjured control (open bars, N=6) and injured (closed bars, N=6) cultures, M ± SEM. Asterisk (*) with solid line indicates significantly different from BSLN (p<0.05); †significantly different from uninjured control at each time point (p<0.05).

**PR39 suppresses NF-κB p65 subunit transactivation following scratch-injury**

PR39 is a specific inhibitor of classical NF-κB pathway activation that functions by selectively binding the α7 subunit of the 20S proteasome and blocks Ikβά degradation, which in turn, prevents transactivation of the p65/p50 heterodimer to the nucleus (27). To
test the hypothesis that targeted pharmacological blockade using PR39 would suppress p65 activation, myotube cultures were treated with 1μm PR39 at 1h prior to initiating each experiment and then subjected to scratch-injury (Figure 5.6.). A significant main effect for time (p<0.01), treatment (p<0.01), and treatment x time interaction (p<0.01) was observed in myotube cultures pre- to post-injury. Untreated injured cultures displayed a variable temporal pattern of p65 activation which increased by 333±26% over 24h and was significantly (p<0.001) different from BSLN by 1h. In response to PR39-treatment, injured cultures displayed a similar temporal pattern of p65 activation and transiently increased by 1041±25% over 24h and was significantly (p<0.01) different from BSLN at 1h and 6-24h. Compared to untreated injured cultures, PR39 transiently reduced p65 activation by 70±35% (p<0.01) from BSLN-3h, but was not statistically different from untreated injured cultures at 6-24h. Those data indicate that PR39 transiently reduced p65 transactivation within the first 3h post-injury, and p65 activation subsequently increased in a similar pattern to untreated cultures until 24h.
Figure 5.6. Effects of scratch injury on NF-kB/p65 transactivation on untreated injured cultures (black bars, N=3) and on PR39-treated injured cultures (grey bars, N=3) at BSLN and 1-24h post-injury, M ± SEM. Asterisk (*) with solid line indicates significantly different from BSLN (p<.05); †significantly different from untreated injured condition at each time point (p<.05).

PR39 suppresses MCP-1 secretion from injured myotube cultures following scratch-injury

To test the hypothesis that PR39-directed blockade of p65 activation would in turn alter MCP-1 protein secretion, control and injured myotube cultures were treated with 1μm PR39 or vehicle control containing an equal volume of PBS at 1h prior to initiating each experiment and then subjected to scratch-injury. A significant main effect for time (p<0.001), treatment (p<0.001), and treatment x time interaction (p<0.001) was observed pre- to post-injury (Figure 5.7.). In control cultures, PR39 stimulated a 1,322±1% increase in MCP-1 secretion over 24h, which was significantly (p<0.001) different from BSLN by 1h. Those data indicate that PR39 stimulated MCP-1 secretion from myotube cultures under normal conditions. In contrast, PR39 stimulated an altered
temporal pattern of MCP-1 secretion from injured cultures, which increased by 489±1% over the first 12h and was significantly (p<0.001) different from BSLN by 1h. Thereafter, MCP-1 secretion only increased by an additional 5% (i.e. 494±3% from BSLN) by 24h, which was not statistically different from secretion at 12h post-injury. Those data indicate that PR39 blunted MCP-1 secretion from myotube cultures following scratch injury. Compared to PR39-treated controls, MCP-1 secretion from injured cultures was reduced by 58±3% over 24h and was significantly (p<0.001) different from control cultures by 1h, demonstrating that pharmacological suppression of NF-kB activation drastically reduced MCP-1 secretion from muscle cells after injury.

To further examine the effects of PR39 on MCP-1 secretion from myotube cultures, fold changes were calculated within each treatment condition (i.e. fold change between untreated control versus injured cultures, and fold change between PR39-treated control versus injured cultures). Cross-sectional analyses were performed at each time point to determine significant differences between treatment conditions as shown in Figure 5.8. In untreated cultures, scratch-injury induced a significant (p<0.05), yet small upregulation of MCP-1 secretion at 1h (0.3±0.2-fold), followed by a 2.2±2.2-fold increase in MCP-1 secretion at 12h (peak, p<0.01), which declined thereafter but remained elevated by 0.6±0.8-fold at 24h (p<0.01). Those data indicate that relative to controls, MCP-1 secretion from injured myotube cultures reached maximum concentrations (C_{max}) at ~12h post-injury and declined thereafter until 24h. In contrast, MCP-1 secretion from PR39-treated injured cultures was significantly (p<0.04) downregulated by 0.3±0.01-fold from 1-24h, relative to PR39-treated uninjured cultures. Compared to untreated myotube cultures, MCP-1 secretion was significantly (p<0.05)
downregulated by 2.9±0.04-fold at 12h and 1.2±0.01-fold at 24h. Taken together, the data presented in Figures 5.7., and 5.8., demonstrate that PR39 significantly altered the kinetics of expression and secretion of MCP-1 from control cultures, grown under normal conditions, as well as from myotube cultures subjected to scratch-injury within 24h.

**Figure 5.7.** Effects of scratch injury and PR39-treatment on uninjured control cultures (light grey bars, N=3) and on injured cultures (dark grey bars, N=3) at BSLN and 1-24h post-injury, mean ± SEM. Asterisk (*) with solid line indicates significantly different from BSLN (p<0.05); †significantly different from untreated injured condition at each time point (p<0.05).
**Figure 5.8.** Comparisons of Fold change between untreated control versus injured cultures (open bars, N=6) with Fold change between PR39-treated control versus injured cultures (black bars, N=3) at BSLN and 1-24h post-injury, M ± SEM. *Significant fold change within conditions at each time point (p<0.05); †significantly difference between untreated condition vs. PR39-treated condition at each time point (p<0.05).

### Scratch-injury induces satellite cell proliferation and PR39 suppresses proliferation

Typical experimental indices of muscle repair are satellite cell proliferation and fusion with myotubes, which comprise the skeletal muscle regeneration program. To determine the basal myogenic potential in normal myotube cultures and the magnitude of the regenerative response (i.e. satellite cell proliferation) to acute-scratch injury, cultures were collected at 19h and 72h later and examined for BrdU$^+$ nuclei, which indicated the presence of proliferating satellite cells in myotube cultures. The number of BrdU$^+$ cells was then expressed relative to the total number of nuclei present in each image. In control cultures, BrdU$^+$ nuclei accounted for 55±12% of total nuclei at 19h and 57±7% of total nuclei at 72h, indicating high myogenic potential in normal C2C12 myotube cultures. In the injured cultures, BrdU$^+$ nuclei accounted for 84±15% of total nuclei at 19h and
77±14% of total nuclei at 72h post-injury. Compared to normal cultures, proliferation increased by 29±13% at 19h and by 20±11% at 72h post-injury, demonstrating that scratch-injury induced a significant (p<0.03) regenerative response (Figure 5.9.B) in my model.

To test the hypothesis that changes in NF-kB activation would associate with muscle repair indices, myotube cultures were treated with 1μm PR39, incubated with BrdU, and then subjected to scratch-injury. PR39-treated controls displayed a 21±16% decrease in proliferation at 19h and an 18±5% decrease in proliferation at 72h, indicating that PR39 significantly (p<0.03) reduced satellite cell proliferation, relative to untreated controls, under normal conditions at each time point. PR39 significantly (p<0.05) reduced the rate of proliferation by 16±12% at 19h, but had no significant effect at 72h post injury. Those data indicate that pharmacological blockade of NF-kB activation significantly reduced the myogenic potential of normal myotube cultures, and the regenerative response to muscle injury; thus, contributing further in vitro evidence that NF-kB is a negative regulator of myogenesis and post-injury regeneration.
**Figure 5.9.** Effects of acute injury and blockade of NF-κB transactivation on myoblast proliferation. A. Representative images at 10X magnification of BrdU⁺ nuclei (top and bottom panels) merged with corresponding phase-contrast images (bottom panels) from untreated control (con) and injured (inj) cultures (left and middle panels) and PR39-treated injured cultures (right panels) at 19 hours post. Myotube cultures were incubated with BrdU loading buffer and either 1μM PR39 or vehicle control (equal vol. of PBS) and then subjected to scratch-injury and immunolabeled with α-BrdU antibody and nuclei stained with DAPI. B. Inhibition of NF-κB transactivation with PR39 restricts myoblast proliferation at 19h (left) and 72h (right) post-injury. The number of BrdU⁺ nuclei are expressed as the percentage of total nuclei from both mononucleated and multinucleated cells. Results are shown as mean ± SEM of 3 independent experiments. *Indicates p<0.05 control vs. injured; † indicates p<0.05 untreated vs. PR39-treated.
Preliminary data: effects of scratch-injury and PR39 on satellite cell fusion

As an exploratory aim, I examined satellite cell fusion with myotubes in both control and injured cultures in the presence and absence of PR39 as illustrated in Figure 5.10.A-B. Satellite cell fusion was determined by manually scoring the total number of BrdU\(^+\) nuclei within the boundaries of myotubes in each image field, using the same images that were used to determine the proliferation index illustrated above in Figure 5.9.A-B. I observed similar total numbers of BrdU\(^+\) nuclei fused with myotubes in control cultures at 19h (26±4.2) and 72h (28±1.4). However, I observed appreciably fewer BrdU\(^+\) nuclei in the injured conditions at 18h and 72, and treatment with PR39 appeared to potentiate that effect but there were no statistical differences between treatment conditions. Therefore, at this juncture, I believe that more replicates of this experiment will be needed.

I made several qualitative observations that I believe are worthy of discussion. In control cultures, activated satellite cells appeared to associate (i.e. lay in close proximity to myotube borders) around the myotubes, but I did not observe any distinct patterns of satellite cell association with myotubes that suggested migration toward and/or fusion with myotubes. In contrast, activated satellite cells in the injured cultures clearly migrated into and accumulated within the wound regions created by scratch-injury. Interestingly, the injured myotube cultures displayed a large amount of newly formed BrdU\(^+\) satellite cells that were closely in contact with myotubes compared with uninjured control cultures (bottom right, panel 5.10.). Although I consistently observed a large number of BrdU\(^+\) nuclei, it was quite rare to see them fused with differentiated myotubes in the injured conditions. This observation suggests that in this particular model of
muscle injury, satellite cells are clearly activated but the injury itself does not induce significant satellite cell fusion with injured myotubes within the 72h experimental timeline. It is plausible that injured myotubes secrete soluble factors that recruit and activate satellite cells, and induce the migration of satellite cells toward the injured myotubes and facilitate repair via autocrine/paracrine signaling of cytokines or growth factors. Upon careful inspection of the control images (top left panel), you can see the nuclei labeled with the white arrows appear dimmer than the nuclei outside of the myotube boundaries. In either case, I will need to continue this work to confirm whether or not that is an accurate interpretation.
**Figure 5.10.** Effects of scratch-injury and blockade of NF-κB transactivation on satellite cell fusion with myotubes. A. Representative images at 20X magnification of BrdU⁺ nuclei (top and bottom panels) merged with corresponding phase-contrast images (bottom panels) from PR39-treated control [(con), left panels] and injured [(inj), right panels] cultures at 19 hours post. White arrows indicate representative BrdU⁺ nuclei incorporated into myotubes. B. Inhibition of NF-κB transactivation with PR39 does not influence satellite cell fusion with injured myotubes at 19h (left) and 72 h (right) post-injury. Results indicate the mean number of BrdU⁺ nuclei within myotubes ± SEM of 3 independent experiments. * indicates p<.05 control vs. injured.
NF-κB activation strongly correlates to MCP-1 secretion following myotube injury

To determine the association between NF-κB/p65 transactivation and MCP-1 secretion following injury, correlations (Pearson’s r) were calculated for the changes in NF-κB activation and MCP-1 secretion in injured myotube cultures, relative to baseline (Figures 5.11.A-C). Cross sectional analyses were then performed between treatment conditions (untreated versus PR39-treated) at each time point to determine if blockade of NF-κB transactivation induced significant changes in NFκB and MCP-1 kinetics in response to injury (Figures 5.11.B-C). Among untreated cultures, NF-κB activation strongly correlated (R=0.69, p<0.01) with MCP-1 secretion from injured myotube cultures relative to baseline (Figure 5.11.A), indicating similar overall kinetic changes in response to injury. Those changes were likely driven by the similar increases in NF-κB and MCP-1 toward peak values from 6-24h post injury (Figures 5.11.B-C).

PR39-treatment significantly (p<0.001) attenuated NF-κB transactivation at 1h (-40±42%) and 3h (-106±61%) post-injury (Figure 5.11.B), leading to a significant (p<0.001) 418±35% increase toward maximal MCP-1 secretion from 1-12h, which was then sustained until 24h (Figure 5.11.C). PR39 significantly (p<0.001) attenuated (-33±3.7%) the peak in MCP-1 secretion at 24h, relative to untreated cultures, demonstrating that blockade of NF-κB transactivation altered the kinetics of both NF-κB and MCP-1 after injury. Moreover, NF-κB and MCP-1 remained strongly correlated (R=0.74, p<0.0001) after PR39-treatment, which suggests that NFκB transactivation and MCP-1 secretion in response to injury are highly interdependent processes.
Figure 5.11. Correlations between changes (Δ) in p65 transactivation and MCP-1 secretion from baseline (BSLN)-24 hours (H) post-injury among injured myotube cultures. Correlations (Pearson’s r) were run within Untreated (blue markers & trend lines) and PR39-treated (red markers & trend lines) conditions; *indicates significant (p<0.01) correlation of p65 (N=3) to MCP-1 (N=3). The correlations of p65 and MCP-1 presented in Figure 5.11.A were derived from the data illustrated in Figures 5.11.B and C. (B) Δ p65 activation in untreated and PR39-treated cultures post-injury. (C) Δ MCP-1 secretion in untreated and PR39-treated cultures post-injury. †indicates significant difference between groups (p<0.05).
Discussion

The purposes of this study were to determine the roles for NF-kB activation and MCP-1 secretion in post-injury muscle repair, and to determine the relationship between NF-κB activation and MCP-1 expression in muscle cells in response to an acute lacerative injury. The classical NF-kB pathway, typically represented by the p65 subunit of the p65/p50 heterodimer complex, is a well-studied regulator of skeletal myogenesis (4, 5, 109), which by definition refers to satellite cell differentiation into myotubes. Myogenesis is regulated by transcription factors such as myogenin, MyoD, and Myf5, which direct myoblasts to exit the cell cycle and fuse together to form multinucleated myotubes (88). Myogenesis typically refers to the early stages of muscle development; however, myogenesis is largely recapitulated in adult muscle regeneration after injury (109). Muscle injury induces a regenerative response by skeletal muscle cells which involves the activation and proliferation of the muscle-resident satellite cell pool, followed by satellite cell differentiation and fusion with injured myofibers.

Post-injury muscle regeneration begins with a degenerative phase characterized by necrosis and infiltration of phagocytic neutrophils, monocytes, and macrophages which remove debris and contribute to the acute-phase inflammatory response via secretion of reactive oxygen/nitrogen species (RONs) and pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), and interferon gamma (IFNγ) ((60, 62, 102). The destructive processes that occur during this phase of regeneration pave the way for the subsequent muscle repair phase characterized by satellite cell activation, proliferation, and fusion with damaged myofibers to facilitate muscle repair. However, the pro-inflammatory/regenerative processes, which are largely driven by immune cell
populations, also contribute to secondary muscle injury to bystander fibers, can delay subsequent muscle repair and promote muscle pathologies such as muscular dystrophy (2, 108). Therefore, identifying the specific molecular pathways and processes responsible for the recruitment of muscle damaging immune cells has become an area of significant importance to human health and disease prevention.

The inflammatory and regenerative responses to acute skeletal muscle injury have been previously described as interdependent processes that govern the rate of muscle repair. However, the underlying molecular pathways and mechanisms that drive inflammation and regeneration are poorly understood. Recent studies have implicated NF-kB activation and MCP-1 protein expression as two signaling molecules that can drive these processes (36, 38, 107); however, their individual roles and interdependence have not been fully determined.

In the present study, NFkB/p65 was expressed in both mononucleated satellite cells and multinucleated myotubes within the cultures at all time points examined. At the anatomical level, distinct differences in the cellular localization within either the cytoplasm or perinuclear space, or within the nuclear compartments of both satellite cells and myotubes were not clearly distinguishable. However, there appeared to be a higher level of constitutive nuclear p65 abundance in the myoblasts compared with myotubes within the uninjured cultures, which is in line with the established role of the classical NF-kB pathway during myogenesis (5).

MCP-1 has a well-characterized roles in the recruitment of immune cells during the inflammatory response to muscle injury (75, 78, 81, 92, 115). However, data on alternative roles of MCP-1 in skeletal muscle are generally lacking from the literature. In
In the present study, intracellular MCP-1 protein levels were significantly (p < 0.04) decreased by 39±15% at 1-24h relative to BSLN in uninjured controls, indicating that under normal conditions, muscle cells decreased their production of MCP-1 protein. Furthermore, MCP-1 secretion from uninjured muscle cells significantly increased by 149% at 12h and by 576% at 24h, which was in opposition to the observed decrease in cytosolic MCP-1 levels. These findings were unexpected from normal myotube cultures as the only perturbation they underwent was replacement of DM 3h prior to initiating the experiment. However, in human primary skeletal muscle, MCP-1 has been shown to negatively regulate insulin signaling and to directly inhibit glucose uptake at physiological MCP-1 concentrations (i.e. < 200pg/ml) (91). Those data suggested an under-reported role for MCP-1 in muscle cell metabolism by regulation of glucose uptake. In relation to the present study, the prolonged decrease in cytosolic MCP-1 and subsequent increase in MCP-1 secretion from normal myotube cultures at 12-24h may be a mechanism to regulate glucose uptake between feeding periods as nutrient levels in the media degrade over time.

In response to injury, I observed a transient increase in cytosolic MCP-1 protein at 1-6h, followed by subsequent increases in MCP-1 secretion at 12-24h post-injury. Those data demonstrate that myotube cultures rapidly increased de novo synthesis and secretion of MCP-1 in response to acute injury, despite an approximate 10% decrease in cell number due to the scratch-injury. Others have reported similar increases in MCP-1 protein levels following mechanical overload-induced injury (78) scratch-injury (105) as well as in animal models (45) Those studies demonstrated that MCP-1 secretion from skeletal muscle functions to recruit inflammatory immune cells and to promote wound
closure and satellite cell proliferation. The present study corroborates previous data and provides novel evidence that skeletal muscle cells themselves produce (within 3h) and secrete (within 12h) a significant amount of MCP-1 during the first 6-24h post-injury. This time period has been well-characterized as the acute phase of inflammation (7, 20) and degenerative phase of regeneration (3, 46). Using a similar in vitro injury model in C\textsubscript{2}C\textsubscript{12} myoblasts, Yahaoui et al. (115) showed that treatment of wounded C\textsubscript{2}C\textsubscript{12} cultures with exogenous MCP-1 accelerated wound closure by downregulating expression of myogenin, leading to satellite cell proliferation within the first 24h post-injury. The decrease in myogenin expression was mediated by ERK 1/2, which promotes the expression of MCP-1.

There appears to be a temporal delay between NFkB transactivation and MCP-1 secretion from C\textsubscript{2}C\textsubscript{12} myotube cultures in response to acute injury, as evidenced by the increase in MCP-1 secretion that emerged at ~6h and reached significance at 12-24h post-injury. PR39 significantly downregulated p65 transactivation from BSLN-12h post-injury, which in turn, resulted in significant and lasting downregulation of MCP-1 secretion from 1-24h post injury. However, it is acknowledged that with any pharmacological approach, the potential for off-target effects that may alter the expression of other regulatory factors or signaling pathways related to NF-kB cannot be completely eliminated. The biochemical data (Figures 5.12.B-C) illustrating the normalized kinetic profiles of NF-kB and MCP-1 are supported first by the strong correlation between p65 activation and MCP-1 expression in untreated cultures, and second, by the fact that p65 and MCP-1 levels remained moderately correlated (in the same direction) in the presence of PR39. Therefore, this work provides strong in vitro
evidence of the interdependence between NFkB/p65 activation and MCP-1 protein secretion from muscle cells in response to injury.

**Conclusion**

This study provided novel insights into the kinetic profiles of NF-kB activation and MCP-1 protein expression and secretion following acute injury. The data suggest that NF-kB and MCP-1 play independent and interdependent roles in the regenerative response to injury. NF-kB inhibition restricted satellite cell proliferation and altered the kinetics of MCP-1 secretion, demonstrating that NF-kB is an important link between inflammatory signaling through MCP-1 secretion and muscle regeneration through its regulation of satellite cell fusion in response to injury. The data from this study have important implications for development of therapies designed to target inflammation or improve muscle repair.
CHAPTER VI

SUMMARY

The overall goal of this dissertation was to examine inflammatory and regenerative responses to acute skeletal muscle damage and to define molecular mediators of repair. In dissertation Study I, I examined the effects of quercetin supplementation on the response to exercise-induced muscle injury in humans. The exercise protocol resulted in a moderate injury, characterized by muscle soreness, prolonged strength-loss, local swelling, and CK release in the blood. Although quercetin did not significantly attenuate changes in those markers of muscle damage, there appeared to be a trend toward a reduction in local muscle swelling at 120 h post-exercise, coinciding with the chronic inflammatory response. Those findings stimulated my interest in exploring the molecular underpinnings that drive muscle repair and local inflammation after injury. Moreover, the results from Study I silenced debate on whether quercetin supplementation in healthy humans has beneficial effects on recovery from muscle-damaging exercise, and had a significant impact on Department of Defense policy for incorporation of quercetin into components of the military rations.

Upon further review of the literature, I found NF-κB and MCP-1 signaling are positioned to be key regulators of muscle repair and inflammation, especially regarding their influences on the interaction between muscle cells and macrophages. In addition, several in vitro studies have implicated quercetin as an inhibitor of both NF-κB activation and MCP-1 synthesis in macrophages, which are known to promote muscle inflammation, remodeling, and repair after injury. However, little is known about the mechanisms by which NF-κB and MCP-1 contribute to muscle and macrophage
responses to injury and if quercetin may modulate those responses. The latter may provide clues to the finding in Study I that quercetin may reduce the local inflammatory response as determined by a reduction in upper-arm swelling in response to eccentric exercise. Thus, using an in vitro approach, I explored the roles of and interdependence of NF-κB and MCP-1 signaling after acute-injury of C2C12 myotube cultures.

The goal of my second study was to explore the specific roles of NF-κB activation and MCP-1 expression within skeletal muscle cells following acute injury. Using an isolated in vitro scratch-injury model of cultured C2C12 myotube cultures (containing both satellite cells and myotubes), I explored: 1) NF-κB activation and MCP-1 protein expression and secretion from injured myotube cultures to determine their specific roles and; 2) the interplay between NF-kB activation and MCP-1 expression in the inflammatory and regenerative responses to injury.

The key findings from Study II were that muscle injury reduced (rather than induced) NF-κB activation at 6-12h, which may be related to the subsequent increase in satellite cell proliferation at 19h and 72h. Blockade of NF-κB activation with PR39 potentiated the effect of injury on NF-kB by diminishing its activation within the first 3h, which resulted in even greater satellite cell proliferation at 19h and 72h, supporting a primary role for NF-kB in muscle regeneration. Muscle injury caused rapid upregulation of cytosolic MCP-1 protein expression at 1-6h, leading to subsequent increases in MCP-1 secretion from muscle cells by 12-24h post-injury, which confirmed a primary role for MCP-1 in the inflammatory response to muscle-injury. Furthermore, PR39 had a profound and lasting effect on MCP-1 secretion, which was reduced from 1-24h post-injury. The effect of PR39 on MCP-1 protein levels may have been attributed to the
transient inhibition of the proteosome. Those data suggested interdependence between NF-kB activation and subsequent MCP-1 protein expression, as well as a secondary role for NF-kB in muscle inflammation as an upstream regulator of MCP-1 secretion. To further examine this interdependence, correlations were applied to the dataset to determine if the changes in NF-kB activation were associated with changes in MCP-1 secretion in response to the injury, and in response to PR39. As expected, NF-kB activation strongly correlated to MCP-1 expression in response to injury, and PR39 increased the correlation coefficient, indicating a high potential for interdependence between NF-kB activation and MCP-1 secretion.

In dissertation Study II, I further characterized the roles of NF-kB activation and MCP-1 expression in regulating the inflammatory and regenerative responses of injured muscle cells to acute injury. Gaining a better understanding of the underlying events that drive the inflammatory response to muscle injury, specifically regarding the interaction between inflammatory and regenerative processes, may lead to new strategies to accelerate restoration of muscle function and recovery from traumatic injury.
APPENDIX A

INFORMED CONSENT STUDY I

Department of Kinesiology
University of Massachusetts
Amherst, MA 01003

Title: Clinical Trial Studies on Quercetin in Healthy Human Subjects: Effects of Quercetin Supplementation on Muscle Soreness and Recovery

Principal Investigator: Priscilla M. Clarkson, PhD, 413-577-3902
Student Investigator: Kevin O’Fallon, Bs, 413-545-6072
Medical Monitor: Pierre Rouzier, MD, 413-577-5146
Sponsor: U.S. Army

Purpose:

Quercetin is a natural compound that is commonly found in onions and green tea that may have antioxidant and anti-inflammatory activities. It is currently commercially available as a dietary supplement and it is thought that quercetin might reduce the stress of exercise. The purpose of this study is to evaluate whether quercetin supplementation will reduce muscle soreness, swelling and inflammation, losses of strength and range of motion, and improve recovery when exposed to strenuous exercise of the biceps muscles. By participating, you will help us determine if quercetin reduces the stress of exercise and improves recovery in the days following an exercise session. The exercise that will be used is an exercise of the biceps muscle (the muscle at the top of your arm, directly above the elbow). The exercise is designed to cause muscle soreness 1 to 2 days after exercise and an increase in muscle proteins in the blood.
Randomization:

In this study, you will be randomly assigned to take quercetin or a placebo and to the arm you will perform the exercises with first (dominant or non-dominant). Before the study begins, you will be assigned a subject identification number. The investigators will enter your identification number into a computer program and the computer will decide if you will take quercetin or a placebo and which arm you will exercise first. You will not know which group you have been assigned to.

Supplementation:

You will be asked to take quercetin or a placebo in the form of a cran-rasberry flavored nutrition bar (similar to a powerbar). You will be provided with either 500mg of orally administered quercetin in the form of a nutrition bar (treatment) or just a nutrition bar that does not contain quercetin (placebo). If you are in the quercetin group, you will take a total of 1000mg quercetin in two 500mg doses each day for 14 days. 1000mg per day is the recommended dose of quercetin that is currently commercially available. The number of research subjects to be tested in this study is 30 in total and will take place January-August 2009. 15 subjects per group with an age range of 18-25 years will be examined. You will be tested here at the University of Massachusetts, Amherst in the Muscle Biology and Imaging Laboratory. Your participation in this study will last 4 weeks.

Eligibility and Requirements for the study: To be eligible for this study you should be between 18-25 years of age and be in good health as determined from a brief health history questionnaire. You must not be training for sporting events which you compete against others, not be taking
dietary supplements (other than vitamin/mineral supplements with 100% or less than the daily recommended allowances (DRI), and not be following a fad diet plan. The study staff will discuss these and any other reasons why you may not be allowed in the study.

Inclusion Criteria:

You must:

1) confirm that all responses provided on the telephone screen are still accurate,

2) be between 18 and 25 years of age,

3) be willing to comply with all study conditions,

4) have at LEAST one readily accessible blood vessel on one or both arms to obtain blood samples from,

5) be willing to refrain from any strenuous or new physical activities while participating in this study,

6) agree to refrain from using oral and topical analgesics, heat or cold treatment (including therapeutic hot showers and baths), physical therapy, massage or any other muscle treatment regimen during the course of the study,

7) agree to refrain from consuming alcohol-containing products and cough/cold products during the study period of Visits 3-8 and Visits 9-15.

8) agree to refrain from consuming any quercetin containing products and vitamin/mineral supplements during the study period of Visits 3-8 and Visits 9-15.

9) be able to understand the study and give written informed consent.
Exclusion criteria: You must not:

1) have an occupation requiring heavy weight lifting or have participated in weight training activity of the upper body within the past 6 months that may influence the response to study exercise as determined by a scale of known intensities (MET scale) for occupation, recreational, and activities of daily living,

2) have undergone orthopedic surgery in the arm (unless cleared by a physician) or have any skeletal, muscular or neuromuscular dysfunction

3) have participated in a muscle soreness trial within the previous 6 months using the arms,

4) be likely to have problems successfully completing the study exercise requirements as determined by the investigator or the study staff; or comprehend the rating scales,

5) be currently taking any medication that would interfere with the study results such as products with analgesic properties as labeled,

7) be using or have used any corticosteroids within the past 8 weeks including topical preparations,

8) be currently taking any medication that would interfere with the study results such dietary supplements containing quercetin or Vitamin C or over-the-counter cold remedies containing quercetin and/or Vitamin C,

9) be taking any therapeutic dietary supplements (other than a vitamin and mineral supplement with ≤100% of the RDA) such as products containing quercetin, high protein supplements designed to increase muscle mass, supplements designed for weight loss, or supplements containing stimulants such as ephedra,

10) be consuming any narcotic preparation (e.g. codeine) or illicit drugs (such as marijuana, etc.) within the previous 7 days,
11) be pregnant or nursing prior to entry into the study,
12) be anemic (as determined by a blood test),
13) be taking blood thinning medications like (Coumadin, Jantoven, Marevan, and Waran),
14) have metabolic disease (diabetes) or an eating disorder (anorexia or bulimia).
15) have a history of dizziness or fainting, fear of needles or the sight of blood during a blood draw procedure.

During the study it is very important that you communicate with the investigators about your health status. You must report any health problems to us immediately by calling the study phone number, regardless of whether or not you think it is directly related to the study.

If you choose to participate and meet the eligibility criteria for the study, you will be required to attend all 15 visits in order to complete the study. However, your participation in this study is completely voluntary. If at anytime you feel uncomfortable with the study procedures and wish to withdraw your participation, you are free to do so without prejudice. The timetable of the study events is summarized in Table 1.
Study Procedures: The following procedures will be used in the study:

Muscle Soreness Assessment: You will be asked to evaluate your level of soreness or pain in your arm using a paper and pencil test while lifting and lowering a 1 or 2 kg weight. Two bicep extensions will be used to assess arm soreness.

Profile of Mood States (POMS) Survey: You will be asked to rate your mood (how you feel) each day of the study on a POMS survey. The POMS survey will help the investigators understand how your mood influences your perception of muscle soreness. The investigators will explain how to use the survey in detail during Visit 1.

Isometric Strength: The isometric strength of each arm will be measured with the arm positioned on the preacher bench (exercise machine) with the elbow fixed at 90 degrees. You will be asked to pull maximally against a fixed resistance for 3 seconds and then rest for 1 minute. You will be asked to do this 2-4 times with each arm.

Isokinetic Strength: The isokinetic torque of each arm will be measured on a Biodex dynamometer (exercise machine). For this test, you will be asked to pull and push maximally against a bar on the machine while it flexes and extends your arm. The bar will flex and extend your arm a total of 12 times (24 contractions in total).
Arm Circumference: The circumference of your arm (biceps) will be measured while your arm is resting at your side and again while you flex your arm at a 90 degree angle (like a waiter holds a tray of food). A mark will be placed on your arm with indelible ink for consistent measurement day to day. Arm circumference will be measured with a spring loaded tape measure.

Range of Motion: Elbow range of motion will be determined by measuring the angle about the elbow as your arm hangs at rest by your side and again while you fully contract your biceps muscle.

Exercise: At each exercise session, you will exercise only one arm. At Visit 1, the arm to be exercised will be chosen in random order. At Visit 10, you will exercise the arm that was NOT exercised at Visit 3. The exercise session will consist of 24 maximal eccentric contractions of the elbow flexors. Each action will be approximately 5 seconds in duration and will be repeated every 15 seconds. To perform the exercise, you will be seated in a modified preacher curl bench with your exercise arm resting on a padded support, the wrist fixed between two padded rollers of the exercise lever and your forearm in the fully flexed position. For each eccentric action, the investigator will pull down on the lever, forcing your forearm into a fully extended position as you exert maximum resistance. You will repeat each action 24 times with 15 seconds rest in between. Immediately following the exercise, your muscle strength will be reassessed.
Physical Activity Monitoring: You will be asked to wear a small activity monitor on your waist during the study (Visits 2-15) to assess your physical activity levels. The investigators will describe in detail when it is okay to remove your activity monitor (e.g. to bathe or sleep) during the study.

Blood Samples: A blood sample will be taken from the vein at the inside of your elbow joint. Approximately 1-2 tablespoons (~12ml) of blood will be taken each time your blood is drawn. There will be a total number of 12 blood draws during this study. Prior to your enrollment in this study, the graduate student study representative, who is trained in blood drawing techniques will counsel you on the possible risks associated with blood drawing and examine your arms (at the elbow joint) to ensure you have at least one accessible blood vessel on each arm to obtain blood samples from. If you do not have at least 1 readily accessible blood vessel, have a history of fainting or dizziness during a blood draw procedure, or are fearful of needles or the blood draw procedure as a whole, you will not be eligible to participate in this study. If you decide during the study that you would no longer wish to have your blood drawn, you are free to withdraw from the study at any time without prejudice.

Schedule:

The study will consist of 3 phases:

Phase 1 - Subjects will wear an activity monitor, complete daily POMS survey, exercise one arm, and criterion measures will be assessed.
Phase 2 - Subjects will take the supplement/placebo twice daily for 7 days, wear an activity monitor, and complete daily POMS survey.

Phase 3 - Subjects will exercise, continue to take supplement/placebo twice daily for 4 days post exercise (11 days in total), wear an activity monitor, and complete daily POMS survey.

Table 1: Timetable of Events

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<thead>
<tr>
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<th>Visit 1</th>
<th>Informed consent document administered</th>
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<tbody>
<tr>
<td>PHASE 1</td>
<td>Visit 2</td>
<td>Baseline testing of criterion measures* (Blood sample not assessed at this visit), assigned an activity monitor (worn)</td>
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<tr>
<td></td>
<td>Visit 3</td>
<td>Criterion measure assessment and blood sample (Arm 1 – dominant/non dominant chosen in balanced order at random)</td>
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<td></td>
<td>Visits 4-8</td>
<td>Criterion measures one arm and blood samples</td>
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<tr>
<td>PHASE 2</td>
<td>Visit 8</td>
<td>Begin quercetin supplementation (7 days pre-exercise, through Visit 15), and continue daily POMS survey</td>
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<tr>
<td>PHASE 3</td>
<td>Visit 9</td>
<td>Baseline testing of criterion measures (Blood sample will</td>
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<td></td>
<td>Visit 10</td>
<td>Criterion measure assessment and blood sample (Arm 2 contralateral arm from day 2)</td>
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<td></td>
<td>Visits 11-15</td>
<td>Criterion measures (Arm 2) and blood samples</td>
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*Criterion measures are POMS survey, soreness, isometric strength, isokinetic strength, range of motion, circumference of the upper arm, and blood sample. (Blood samples will not be assessed on Visit 2 or 9)

Dietary Restrictions: (Visits 3-8 & 10-15)

Prior to starting the study, you will be provided with a list of foods containing quercetin we will ask you to refrain from eating. During the study, we will ask that you do not ingest foods
on the list we provide you until after your final visit (Visit 15). Water will be provided while you are at the laboratory and we will encourage you to drink as much water as you like.

*Overnight Fasting: (Visits 3-8 & 10-15)*

You will be asked to fast overnight for ~12 hours before Visits 3-8 & 10-15 of the study. The longest time you will be without food is during the overnight fast. When you arrive at the lab each morning you will be given ~16 oz. of water prior to blood collection to enhance hydration.

*Standardized Meals: (Visits 3 & 10)*

On Visits 3 & 10, you will be given a standard snack (bagel with cream cheese) immediately after blood collection, before performing your criterion measures. After you have completed your post-exercise strength test, you will be given two First Strike bars to eat. You will be encouraged to drink as much water as you like while you are at the laboratory.

*General Testing Schedule:*

*Visit 1, Informed Consent and Orientation (30 minutes):* You will be asked to review this informed consent document approved by the University of Massachusetts Human Subjects Institutional Review Board. You will receive a copy of this document for your records and a verbal explanation of the study. You will be given as much time as you need to review this informed consent form and we encourage you to ask any questions you have about the study procedures. After you have read this form we will ask you to sign it. You will be provided a copy
of this informed consent form. After signing the informed consent form, you will complete a brief medical history questionnaire and your age, height and weight will be recorded. If you are a woman, we will ask you to take a home pregnancy test (Clear Blue Easy) at this time. We will ask you to provide us with a urine sample so we may determine your pregnancy status. If your pregnancy test is positive, we will provide you with the pregnancy test results and telephone numbers of appropriate agencies on campus to contact if you are interested. If you are pregnant, you will not be able to participate in this study. The medical history questionnaire will ask you if you have been told by a physician within the past 2 years that you were anemic. If so, you will be asked to provide a blood sample to ensure that you are not anemic at the time of the study. You will then be randomized to take either quercetin or placebo and the arm you will exercise during phase 1.

**Visit 2 Baseline Measure Assessment (30 minutes):**

You will report to the laboratory and complete a POMS survey, have your muscle soreness, strength, arm range of motion and circumference measured, and be given an activity monitor to wear during the study. Specific details are described in detail in the “Procedures” section below.

**Visit 3 Baseline Measures, Exercise, Post-Exercise Strength Assessment (60 minutes):**

You will come to the laboratory fasted (no food or drink for 12 hours except water). You will complete a POMS survey, your vital signs will be measured and you will have a blood sample drawn for measurement of quercetin and muscle proteins in the blood and for archiving. On this visit, you will have your muscle soreness, strength, range of motion and arm circumference measured. You will then exercise one arm. You will perform 24 maximal
eccentric contractions of the biceps muscle. Immediately following the exercise the strength of your exercised-arm will be measured and you will complete another POMS survey. The baseline measures and exercise are described in the “Procedures” section below.

**Visits 4-8 (5 consecutive days starting 1 day after Visit 3) (25 minutes each):**

You will come to the laboratory for visits 4-8. You will complete a POMS survey and have a blood sample taken for analysis of quercetin and muscle proteins in the blood and for archiving. You will then have your muscle soreness, strength, range of motion and arm circumference assessed. At Visit 8, you will be issued a 7-day supply of quercetin or placebo.

**Visits 9-15 (repeat of Visits 2-8 above):**

These visits will be the same as Visits 2-8 except with the opposite arm being tested.

**Risks:** The possible risks, discomforts and side effects of the procedures are described below, including safeguards to be used for your protection:

**Blood Samples:** During the blood draws, you may feel pain initially when the needle is inserted and you may develop a bruise where the blood samples are obtained. The pain and bruising are usually mild and subside within a week. To minimize these risks, blood collection procedures will only be performed by trained individuals using sterile technique at all times. There is also a small chance that you could faint during the blood draw but this risk will be minimized by having you lie in a reclining chair during and
immediately following the procedure. The study staff will make every effort to ensure your safe participation in this study and minimize the potential for the above stated risks.

**Quercetin Supplementation:** There are minimal risks associated with oral quercetin use, including rare reports of nausea, headache and mild tingling of the extremities. Quercetin is currently commercially available as a dietary supplement and will only be administered to you within the recommended dosages. 1000mg per day is the recommended dose of quercetin that is currently commercially available as a dietary supplement. In this study, you will ingest 1000mg per day for 14 consecutive days. Quercetin should be avoided by women who are pregnant or nursing. If you are a woman, and you think you have become pregnant during the study, you will need to notify staff immediately and discontinue the use of quercetin.

**Exercise:** You may experience fatigue from the exercise but this feeling should subside within a few hours after the exercise. You may also experience muscle soreness from the exercise but this feeling should subside within approximately 6 days. The extent of the soreness could be such that there is some loss of strength. However, this strength loss should not be enough to prevent daily activities such as driving a car or combing your hair, but it may make them mildly difficult. The risk of serious injury (such as muscle pull or strain) from the exercise is minimal in healthy subjects who have no cardiovascular or musculoskeletal problems or surgery to the arm or the shoulder. In an extremely rare case, there is the possibility that you could tear the tendon that attaches the biceps muscle
to the lower arm bone while doing the exercise. However, a trained study representative will be working with you to minimize this risk. Exercise testing and training can cause irregular heart-beats, heart attack, heart stoppage and even death; all of these are extremely rare.

When you agree to participate in the study, you will be given a cell phone number to call if any problems arise. This number will allow you to reach an investigator 24-hrs/day.

During the study, if you experience any unforeseen health problems, injuries or accidents you will be required to notify study staff immediately, regardless of whether you feel they are related to the study or not.

**Benefits:** Participating in this study will provide no immediate or long-term benefits to you; however, the information gained may have future military and civilian applications. Quercetin has been purported to reduce the harmful effects of free radicals (oxidative-stress) and inflammation after strenuous physical activity, which may improve the recovery time after such activities. These benefits of quercetin could aid military personnel in the field, keeping them more fit to perform necessary duties and increase their survivability on the battlefield. By the same action, quercetin may also improve the response to strenuous activity in civilian athletes and recreational sportsmen.
**Medical Treatment:** The University of Massachusetts does not have a program for compensating subjects for injury or complications related to human subjects’ research but the study personnel will assist you in getting treatment. Medical treatment will be available at the University Health Services for a fee. Investigators will aid you in every way to see that you get proper medical attention. If you get hurt or sick because of this research study, you can receive medical care at an Army hospital or clinic free of charge. You will only be treated for injuries that are directly caused by the research study. The Army will not pay for your transportation to and from the hospital or clinic. If you have questions about this medical care, talk to the principal investigator for this study Dr. Priscilla Clarkson at 413-577-3902. If you pay out-of-pocket for medical care elsewhere for injuries caused by this research study, contact the principal investigator. If the issue cannot be resolved, contact the U.S. Army Medical Research and Materiel Command (USAMRMC) Office of the Staff Judge Advocate (legal office) at (301) 619-7663/2221.

**Enrollment/Length of Study:** It is expected that 30 subjects in total will be enrolled in this study. This study is expected to last for 6 months, but your participation is expected to last for 4 weeks.

**Compensation:** Your compensation upon completion of the study will be $200.00. You will receive full payment only if you complete the entire study. If you choose to drop out of the study prior to completion of visit 2, you will not be compensated. If you drop out
of the study between visits 3-15, you will receive $15.39 per visit for each visit you complete. No compensation will be given if you do not complete up to and including visit 2. Payment will be received within 4-6 weeks of completing the study or from the time you withdrew.

**Archive Sample Ownership:** Some of the blood taken during the study will be saved and stored (archived). The archived samples will strictly be used for extra tests related to the study to understand its results. No other testing, to include genetic or DNA testing will be performed on the samples. Archived samples are the property of the University of Massachusetts Amherst Muscle Biology and Imaging Laboratory. By agreeing to participate in this study, you willingly release your ownership of blood samples to the University of Massachusetts Amherst Muscle Biology & Imaging Laboratory. Furthermore, if you choose to withdraw from the study early, the samples you have provided prior to your withdrawal are considered the property of the University of Massachusetts Amherst Muscle Biology & Imaging Laboratory and will be archived. However, if you wish to have your samples removed from the study, you may submit a request in writing to Dr. Priscilla Clarkson and your samples will be destroyed. Ownership of the samples will not be transferred to other investigators. The samples will be used only by researchers working on this study, and only for the research related to the study described above.

**Confidentiality:** The personal information obtained from this study will be regarded as private and confidential by the investigators. Representatives of the U.S Army Medical
Research and Materiel Command are eligible to review records as a part of their responsibility to protect human subjects in research. All participants will be identified by subject number, and data will be kept in a locked cabinet on a day-to-day basis. It will not be released to anyone except upon your written consent. Your right to privacy will be maintained in any ensuing analysis and presentation of the data. All identifiers (name, address, phone number) will be removed before future presentation and publication of the data.

Voluntary Participation: You may withdraw from this study at any time in writing or by telephone at 413-545-6072 (Investigator’s phone number) and discontinue participation in the study without prejudice or consequence. Your participation is completely voluntary. In the event that you choose to withdraw from the study early, any information collected from you will be kept on file for the duration of the study. If applicable, you will be compensated for the number of visits you completed after Visit 2

Request for additional information: You are encouraged to express any questions, concerns or doubts about this study at any time to Kevin O’Fallon at 413-545-6072. You may also contact the principal investigator Priscilla Clarkson at 413-577-3902. The investigators will attempt to answer all questions to the best of their ability. The investigators fully intend to conduct the study with your best interest, safety, and comfort in mind at all times. If you would like to speak with someone not related to this study, you may contact the university compliance
officer by phone at 413-545-3428 or email at humansubjects@ora.umass.edu. The investigators have read and understood the Assurance of Compliance with OHRP Regulations for Protection of Human Subjects. A copy of this document can be found at http://www.umass.edu/research/humsub.html.

SUBJECT STATEMENT OF VOLUNTARY CONSENT:
When signing this form I am agreeing to voluntarily enter this study. I understand that, by signing this document, I do not waive any of my legal rights. I have had a chance to read this consent form, and it was explained to me in a language which I use and understand. I have had the opportunity to ask questions and have received satisfactory answers. I have been given a copy of this Informed Consent Form.

Subject Name (Print or Type)
________________________________________

Signature Date

STUDY REPRESENTATIVE STATEMENT:
I have explained the purpose of the research, the study procedures, the possible risks and discomforts, the possible benefits, and have answered any questions to the best of my ability.

Study Representative Name (Print or Type) Date

________________________________________

Signature Date
APPENDIX B

UNIVERSITY INSTITUTIONAL REVIEW BOARD APPROVAL LETTER

UNIVERSITY OF MASSACHUSETTS
AMHERST
Human Research Protection Office
Research Administration Building
70 Butterfield Terrace
Amherst, MA 01003-9242

Office of the Vice Chancellor
for Research and Engagement
voice: 413.545.3428
fax: 413.577.1728

University Institutional Review Board (IRB)
Certification of IRB Approval

Date: August 14, 2009
To: Professor Priscilla Clarkson and Kevin O’Fallon, Kinesiology Department
From: Margaret Burggren, Human Protection Administrator, Human Subjects IRB

The University of Massachusetts Amherst Institutional Review Board (IRB) has reviewed the following research protocol in accordance with the UMASS Federalwide Assurance.

Title: Clinical Trial Studies on Quercetin in Healthy Human Subjects: Effects of Quercetin Supplementation on Exertional Muscle Damage, OGCA# 109-0237
Status: APPROVED – Full Board Review on August 6, 2009 for continuing research
Date: This project approval will expire on September 5, 2010.

The IRB approval is granted with the understanding that investigator(s) will do the following:

- **Modifications** – Changes in any aspect of the study (for example, project design, procedures, consent forms, advertising materials, additional key personnel or subject population) are submitted to the IRB for approval before instituting the changes.

- **Consent Forms** – All subjects are given a copy of the consent form. Investigators are required to retain consent documents for six (6) years after close of the grant or three (3) years if unfunded.

- **Training** – Human subject training certificates, including those for any newly added personnel, are provided for all key personnel.

- **Adverse Events/Unanticipated Problems** – All events occurring in the course of the protocol are reported to the IRB as soon as possible, but not later than five (5) working days.

- **Continuing Review** – IRB Protocol Report Forms are submitted annually at least two weeks prior to expiration, six weeks for protocols that required full review.

- **Completion Report** – The IRB is notified when your study is complete. To do this, complete the IRB Protocol Report Form and select “Final Report.”

Please refer to the web page at [http://www.umass.edu/research/comply/humancomp.html](http://www.umass.edu/research/comply/humancomp.html), call the Human Research Protection Office (HRPO) at 5-3428, or e-mail Nancy Swett at nswett@ura.umass.edu with any questions you may have regarding this certification.

c.c. OGCA
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