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Molecular Mechanisms Underlying the Contralateral Repeated Bout Effect (CRBE) in Human Skeletal Muscle

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**MOLECULAR MECHANISMS UNDERLYING THE CONTRALATERAL
REPEATED BOUT EFFECT (CRBE) IN HUMAN SKELETAL MUSCLE**

A Dissertation Presented

by

LING XIN

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

DOCTOR OF PHILOSOPHY

May 2015

Department of Kinesiology

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LING XIN

Approved as to style and content by:

Lawrence M. Schwartz, Chair

Patty Freedson, Member

Sarah Witkowski, Member

Maria Urso, Consulting Member

Patty Freedson, Department Chair
Department of Kinesiology

DEDICATION

This dissertation is dedicated in memory of my former adviser, Dr. Priscilla M. Clarkson, who will be missed by all those who were lucky enough to know her. I also dedicate this work to my entire family including my parents, my brother, my sister, my husband Feng, and my lovely son Raymond.

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First and foremost, I would like to express my special appreciation and thanks to my former advisor Professor Dr. Priscilla M. Clarkson, who has been a tremendous mentor for me. I would like to thank her for her encouragement, patience, motivation, enthusiasm, and immense knowledge. Her advice on both research as well as on my career have been priceless. I have obtained excellent training in the Clarkson laboratory to grow as an independent research scientist.

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ABSTRACT

MOLECULAR MECHANISMS UNDERLYING THE CONTRALATERAL
REPEATED BOUT EFFECT (CRBE) IN HUMAN SKELETAL MUSCLE

MAY 2015

LING XIN, B.S. JIANGXI MEDICAL COLLEGE, CHINA

M.S. SHANGHAI MEDICAL COLLEGE OF FUDAN UNIVERSITY, CHINA

M.S., UNIVERSITY OF MASSACHUSETTS AMHERST

Ph.D., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor Priscilla M. Clarkson and Professor Lawrence M. Schwartz

Eccentric (muscle lengthening) exercise induces temporary muscle damage and excessive inflammation is believed to play a role in the secondary muscle damage after damaging exercise. A bout of eccentric exercise typically leads to an adaptation response such that the same muscle is less susceptible to the damage induced by a subsequent similar exercise bout, a phenomenon known as repeated bout effect (RBE). Conflicting data exist regarding the existence of the contralateral RBE (CRBE) in humans, where exercising one muscle group induced an adaptation in the contralateral muscle group. As a result, little is known about the molecular and cellular mechanisms underlying the CRBE. Therefore, the primary objective of this dissertation was to verify if the CRBE exists in leg muscles and to identify the key genes and signaling pathways that contribute to this phenomenon. Thirty-six men performed 100 maximal eccentric actions of the knee extensors using one leg and repeated the exercise bout with the contralateral leg five weeks later. *Vastus lateralis* muscle biopsies were taken from eccentric-exercised (ECC)

and control (CON) legs at 3 h after each exercise bout and used for all the three studies in this dissertation.

Study I examined the existence of the CRBE in knee muscles and tested if the attenuated induction of nuclear factor-kappa B (NF- κ B; an important regulator of muscle inflammation) following eccentric exercise is a potential mechanism. Subjects displayed a reduced muscle strength loss and a blunted increase in nuclear NF- κ B DNA-binding activity in the contralateral leg after bout 2 compared to bout 1, indicating the existence of the CRBE in leg muscles and the possible involvement of NF- κ B in the CRBE.

Study II was conducted to determine if NF- κ B signaling pathway is a critical regulator for the CRBE. Human whole-genome microarray and follow-up analysis using Ingenuity Pathway Analysis (IPA) software demonstrated that the inflammatory response was one of the top differentially regulated biological functions and identified NF- κ B as a key signaling pathway affected by the CRBE. Quantitative real-time polymerase chain reaction (qRT-PCR) confirmed that the induction of three NF- κ B-related pro-inflammatory genes (*ANKRD1*, *CRY61*, and *IL1R1*) in response to eccentric exercise was attenuated in the contralateral leg in bout 2 compared to bout 1. These results provide the first gene expression pattern of human muscle in the CRBE and strongly suggest that an attenuated inflammatory response mediated by NF- κ B contributes to the CRBE.

Study III tested if ANKRD1 is a potential biomarker for skeletal muscle damage because ANKRD1 displayed the greatest induction following damaging exercise in bout 1 among the differentially expressed genes in the CRBE identified from the microarray analysis in Study II. As shown in Study II, microarray and qRT-PCR demonstrated that ANKRD1 mRNA was up-regulated at 3 h post-eccentric exercise. To analyze ANKRD1

expression at protein level, muscle biopsy samples collected from another study were used. Seven young men performed eccentric exercise with one leg and biopsies were obtained pre-exercise from the CON leg and 24 h post-exercise from the ECC leg. A significant increase was observed in the cytoplasmic ANKRD1 protein levels at 24 h post-eccentric exercise. Most importantly, for the first time, these data indicate that ANKRD1 nuclear accumulation is positively correlated with muscle strength loss. Finally, via Gene Expression Omnibus (GEO) profile data analysis, ANKRD1 mRNA was found to be up-regulated in all of the assayed muscle damage models. The consistent increase in ANKRD1 mRNA across various skeletal muscle damage models, and the positive correlation between ANKRD1 protein and muscle strength loss, suggest that ANKRD1 is a potential muscle damage biomarker.

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CHAPTER 1

INTRODUCTION

Statement of the Problem

Unaccustomed exercise induce transient muscle damage as demonstrated by cellular damage, muscle soreness, and prolonged strength loss (27). This exercise causes initial mechanical damage to the muscle followed by an acute inflammatory response (160) leading to secondary damage. Exercise-induced muscle damage results in an adaptation response, whereby the damaging effects of subsequent exercises are attenuated in the same muscle. For example, a second bout of eccentrically biased exercise produces less damage in a muscle that has been exposed previously to a similar exercise bout, a phenomenon known as the “repeated bout effect” (RBE) (27, 126). To date, only a few studies have reported on the existence of the contralateral RBE (CRBE) in humans, where exercising one muscle group (elbow flexors) induced an adaptation in the contralateral muscle group (48, 54, 122, 155). Given the paucity of studies examining the CRBE, it is not surprising that the underlying mechanisms are not understood.

Our laboratory previously provided direct evidence of an increase in the activation of the transcription factor kappa B (NF- κ B) at 3 h after eccentric exercise in humans (57). Since NF- κ B contributes to the secondary muscle damage after eccentric exercise (24, 74), we hypothesized that attenuation of NF- κ B activation may be involved in the CRBE. Therefore, **the first aim** of this dissertation was to determine if the CRBE exists in leg muscles and to examine a possible role for the NF- κ B pathway in this response. This aim was investigated in Study I and II of this dissertation. In Study I, the existence of the CRBE in the lower limbs was examined in humans, as demonstrated by a

reduction in the anticipated muscle strength loss in the contralateral leg when it was challenged five weeks after damaging exercise in the initial limb. In addition, data are presented from NF- κ B DNA-binding activity in the muscle biopsy samples taken from both the control (CON) and eccentric-exercised leg (ECC) at 3 h after each eccentric exercise bout. These biopsy samples were also used in Study II to examine the transcriptional changes altered by the CRBE, especially the changes in NF- κ B-regulated genes, as well as the phosphorylation level of I κ B α (an upstream regulator of NF- κ B).

In Study II, microarray analysis was utilized to screen for genes that are differentially expressed in the CRBE. The gene that displayed the greatest induction following damaging exercise in bout 1 was *ANKRD1* (fold change, FC = 103.4). With such a rapid and dramatic change in expression following exercise, I sought to determine if the *ANKRD1* gene is induced in other muscle injury models/disorders in mammals and if a change in the ANKRD1 protein is correlated with other muscle damage markers. Therefore, **the second aim of** this dissertation was to determine if ANKRD1 is a potential marker of skeletal muscle damage induced by eccentric exercise and other pathological conditions, which is described in Study III.

Experimental Approach

To address the overall dissertation aims, multiple approaches have been applied to assess the changes of NF- κ B signaling pathway in the muscles after two bouts of eccentric exercise using opposite legs, at both mRNA and protein levels. Chapter 3, 4 and 5 present the details of the corresponding methodologies utilized in Study I, II, and III, respectively. Chapter 3 (Study I) describes a detailed study design, biopsy sample

collection procedure, muscle function and blood sample analysis, and NF- κ B DNA-binding activity assay. Chapter 4 (Study II) presents whole genome microarray technology and follow-up analysis to screen for differentially expressed genes, pathways, functions, and networks, quantitative real-time PCR (qRT-PCR) to confirm changes in the genes of interest, and Western blotting to quantify phosphorylation level of I κ B α . Chapter 5 (Study III) describes the potential of ANKRD1 as a universal muscle damage biomarker via a combination of methodologies including microarray analysis, qRT-PCR, Gene Expression Omnibus (GEO) profiling, Western blotting, and immunohistochemistry.

Aims and Hypotheses

Study I: A Contralateral Repeated Bout Effect Attenuates Induction of NF- κ B DNA-binding Following Eccentric Exercise

Although the original study was designed to examine the effects of botanical supplements on the response to strenuous exercise (See Appendix), there was no significant difference between the supplement and placebo groups (no significant group or group \times bout interaction) as analyzed by a repeated measures analysis of variance (ANOVA). However, subjects demonstrated attenuated strength loss after the repeated exercise bout (significant bout effect) in the contralateral leg, a phenomenon known as the CRBE. Consequently, I examined the existence of the CRBE after two bouts of maximal eccentric exercise (the initial bout with the ipsilateral leg and the second bout five weeks later with the contralateral leg) by examining the alterations in the indirect

exercise-induced muscle damage (EIMD) markers (e.g. muscle strength loss, blood creatine kinase (CK) activity, muscle soreness) after the two exercise bouts. Since NF- κ B contributes to the secondary muscle damage after eccentric exercise (24, 74), I also sought to determine if the attenuation of NF- κ B induction following eccentric exercise is a potential mechanism.

Specific Aim #1: To measure CK activity, muscle soreness, and muscle strength after two bouts of eccentric exercise of knee extensors with the opposite leg.

Hypothesis #1: CK increase, development of muscle soreness, and muscle strength loss will be attenuated in the contralateral leg after bout 2 compared to the ipsilateral leg after bout 1.

Experimental Design: Thirty-six young men performed two bouts of 100 maximal eccentric actions of the knee extensors on each leg. Serum CK activity, muscle soreness and muscle strength were measured on the exercised leg before and for 5 days after exercise bout 1 and bout 2. **Brief Results:** Thirty-one subjects had viable data for the statistical analysis. Muscle strength loss was reduced in bout 2 relative to bout 1. There were no significant differences in CK activity or muscle soreness between the two bouts for post-exercise.

Specific Aim #2: To determine NF- κ B DNA-binding activity in skeletal muscle after two bouts of eccentric exercise of knee extensors using opposite legs.

Hypothesis #2: The anticipated increase of NF- κ B DNA-binding activity will be attenuated in the contralateral leg after bout 2 compared to the ipsilateral leg after bout 1.

Experimental Design: Thirty-six young men performed two bouts of 100 maximal eccentric actions of the knee extensors using opposite legs. *Vastus lateralis* muscle biopsies were taken from both eccentric exercised and control legs 3 h after each exercise bout. Enzyme-linked immunosorbent assay (ELISA)-based analysis was performed to analyze NF- κ B (p65) DNA-binding activity in the muscle biopsy samples collected at 3 h after bout 1 and bout 2.

Brief Results: p65 DNA-binding activity was increased following eccentric exercise (as compared to the control leg) in both bouts. Compared with bout 1, the increase in NF- κ B DNA-binding activity post-exercise was attenuated after bout 2.

Study II: Transcriptome Signature of the Contralateral Repeated Bout Effect (CRBE) in Human Skeletal Muscle

The purpose of this study was to examine the changes of the NF- κ B-mediated inflammatory response after two bouts of eccentric exercise by analyzing up- and down-stream components of the NF- κ B pathway. Analysis of the muscle biopsy samples collected at 3 h post-exercise after two bouts of eccentric exercise of knee extensors (the initial bout with the ipsilateral leg and the second bout five weeks later with the contralateral leg) was performed.

Specific Aim #1: Use whole genome microarrays and subsequent network analysis to screen for genes and signaling pathways that are differentially altered in the CRBE.

Hypothesis #1: The whole genome microarray data analysis will generate the first transcriptome signature of the CRBE and identify NF- κ B as a key signaling pathway affected by the CRBE.

Experimental Design: *Vastus lateralis* muscle biopsies collected from both eccentric exercised and control legs 3 h after each exercise bout in study I were also used for study II. Agilent whole-genome microarrays were utilized to examine gene expression changes and follow up pathway, function, and network analysis was conducted using ingenuity pathway analysis (IPA) software.

Brief Results: Microarray data analysis revealed that 861 transcripts were significantly altered as a result of the CRBE ($P(\text{exercise} \times \text{bout}) < 0.05$), among which, 27 were NF- κ B-related genes. IPA function analysis demonstrated that inflammatory response is a key biological function altered by the CRBE. IPA network analysis showed that NF- κ B was included in the center of the top network, indicating that NF- κ B is a key signaling pathway involved in the CRBE.

Specific Aim #2: To measure the expression of specific NF- κ B-associated genes that encode pro-inflammatory mediators.

Hypothesis: There will be an attenuated increase in the expression of these genes at the mRNA level in the contralateral leg after the second bout compared to the ipsilateral leg after bout 1.

Experimental Design: qRT-PCR was applied to analyze the expression levels of five genes of interest (*CCL2*, *ANKRD1*, *IL1R1*, *CYR61*, and *TGF β 2*) chosen from the microarray data analysis. These genes have been selected for qRT-PCR confirmation

analysis because microarray data showed they are altered in bout 2 compared to bout 1 and they are all NF- κ B-associated genes that encode pro-inflammatory mediators.

Brief Results: All genes selected for analysis were up-regulated post-exercise in both bout 1 and bout 2. Three genes (*ANKRD1*, *IL1R1*, and *CYR61*) demonstrated blunted gene expression responses to eccentric exercise between bouts.

Specific Aim #3: To determine if phosphorylation of the I κ B α is altered after two bouts of exercise using the opposite leg.

Hypothesis #3: There will be an attenuated increase in phosphorylation of I κ B α in the contralateral leg after the second bout compared to the ipsilateral leg after bout 1.

Experimental Design: Western blot analysis was performed to analyze cytoplasmic protein contents of I κ B α and phosphorylated (p- I κ B α).

Brief Results: Neither I κ B α nor p-I κ B α were significantly ($P > 0.05$) altered after exercise in either bout 1 or bout 2.

Study III: Ankyrin Repeat Domain 1 (ANKRD1) Is a Potential Marker of Skeletal Muscle Damage Induced by Eccentric Exercise and Other Pathological Conditions

From the microarray analysis in Study II, I noticed that *ANKRD1* has the highest fold change (FC = 103.4) in expression of any gene following a bout of exercise. The *ANKRD1* gene encodes the ankyrin repeat domain 1 (ANKRD1, also known as cardiac ankyrin repeat protein (CARP)) (25). With such a rapid and dramatic change in expression post-eccentric exercise, I hypothesize that ANKRD1 may represent a useful

biomarker of muscle injury that may provide insights into the molecular mechanisms that mediate responses to damaging exercise. To test this hypothesis, it was necessary to determine if ANKRD1 is induced in other muscle injury models/disorders in mammals. In addition, only a few studies (23, 80, 95) have reported changes in ANKRD1 gene expression in human skeletal muscle after eccentric exercise, and it is not clear if the expression of ANKRD1 protein is also altered after damaging exercise. Since protein is presumably the ultimate functional molecule, it is important to also examine changes in ANKRD1 protein levels after eccentric exercise. Thus, the Specific Aims of study III were:

Specific Aim #1: To determine if ANKRD1 mRNA (gene *ANKRD1*) is induced in human skeletal muscle after eccentric exercise.

Hypothesis #1: Both microarray and qRT-PCR data will display a substantial increase in the gene level of *ANKRD1* at 3 h post-eccentric exercise.

Experimental Design: The data for this aim were obtained from the microarray and qRT-PCR data of Study II. The data were reorganized to show the *ANKRD1* gene expression changes at 3 h post-eccentric exercise.

Brief Results: Microarray data demonstrated that *ANKRD1* was up-regulated 103.4-fold at 3 h post-eccentric exercise, which was confirmed by qRT-PCR (115.7-fold increase).

Specific Aim #2: To measure ANKRD1 protein changes post-eccentric exercise and how the changes are related to muscle strength loss.

Hypothesis #2: ANKRD1 protein will be increased after exercise and its increase is positively correlated to muscle strength loss.

Experimental Design: Seven young men performed eccentric exercise with one leg and biopsies were obtained pre-exercise from the control (CON) leg and 24 h post-exercise from the eccentric exercised (ECC) leg for ANKRD1 protein assessment. Western blot was used to measure cytoplasmic and nuclear ANKRD1 protein content and Pearson's correlation analysis was used to assess the relationship between ANKRD1 protein changes and the peak muscle strength loss. Immunofluorescence microscopy and the image analysis software ImageJ were utilized to quantify and localize ANKRD1 protein staining in the muscle biopsy samples.

Brief Results: Western blot data demonstrated that nuclear ANKRD1 protein levels 24 h post-exercise were positively correlated with peak muscle strength loss post-exercise. Compared to the CON leg, the ECC leg had an increased cytoplasmic ANKRD1 protein at 24 h post-exercise.

Specific Aim #3: To examine if ANKRD1 mRNA level is induced in different mammalian muscle damage models.

Hypothesis #3: *ANKRD1* is significantly increased in the various muscle damage models.

Experimental Design: Using Gene Expression Omnibus (GEO) profile data, I chose five data sets to analyze *ANKRD1* changes: Duchene muscular dystrophy (DMD) patients, mdx mice, freeze-induced muscle injured mice, α 2-laminin deficient muscular dystrophy mice, and infants with Infantile-onset Pompe disease.

Brief Results: ANKRD1 mRNA was significantly ($P < 0.01$) upregulated in all analyzed muscle damage models.

Significance

The innovation of this study is that it, for the first time, proposes molecular and cellular mechanisms to explain the CRBE in humans. Results from Study I and II suggest that the CRBE is due, in part, to an attenuated acute inflammatory response mediated by the NF- κ B pathway, which may have implications for clinical and rehabilitation settings. For example, the identified NF- κ B pathway involved in the CRBE may be important in the development of interventions to maximize the therapeutic benefits of the CRBE when setting up rehabilitation exercise for a unilaterally immobilized limb and in identifying targets of future therapies to facilitate muscle recovery. Moreover, Study III identifies ANKRD1 as a novel muscle damage biomarker induced by eccentric exercise and other pathological conditions.

CHAPTER 2

REVIEW OF LITERATURE

Introduction

Unaccustomed exercises, especially exercises that involve eccentric (muscle lengthening) actions, induce transient muscle damage that is typically manifested by temporary muscle swelling, soreness, and loss of muscle function (27). In 1902, Hough reported muscle soreness after exercise and suggested that muscle soreness was due to microtears in the muscle. Since then, many researchers have attempted to assess exercise-induced muscle damage (EIMD) via direct and/or indirect markers and uncover the mechanisms driving this phenomenon. Although more than 500 published studies have investigated EIMD, the exact mechanisms underlying it are still not well understood.

Exercise-damaged muscle results in an adaptation response. For example, a repeated bout of eccentrically-biased exercise produces less damage in a muscle that has been exposed previously to a similar exercise bout, a phenomenon known as the “repeated bout effect” (RBE) (126). Several theories, including neural, mechanical, and cellular mechanisms, have been proposed to account for RBE (for reviews see (100, 101)). Only a few studies have investigated if RBE also exists in the contralateral muscle group after a bout of eccentric exercise (30, 54, 155). These studies suggested that neural adaptation may be a mechanism driving the contralateral RBE (CRBE) (30, 54, 155). Because of the paucity of evidence regarding the CRBE, the molecular and cellular mechanisms underlying this process are essentially unknown.

The objective of this review is to summarize the literature concerning EIMD and RBE with a special focus on possible molecular and cellular mechanisms underlying

EIMD and the CRBE. I will first summarize the reported evidence for EIMD and describe the direct and indirect markers commonly used to assess EIMD. Second, I will discuss potential molecular and cellular mechanisms driving EIMD. Specifically, a detailed account of the acute inflammatory response after damaging exercise and possible roles of nuclear factor-kappa B (NF- κ B) in this inflammatory response will be discussed. Third, I will describe the evidence regarding the ipsilateral and contralateral RBE and the potential mechanisms driving each. Finally, based on the literature reviewed, I will close this review with a summary of the key concepts and the critical gaps in the literature that I will address in the studies of this dissertation.

Exercise-Induced Muscle damage (EIMD)

Human EIMD models

Muscle can contract in three different ways: isometric, concentric, or eccentric. A contraction during which a muscle produces tension without length change is called an isometric action. If a muscle is shortening while generating force, it is a concentric action. If an external loading force exceeds the maximal force that a muscle can produce, the muscle will be forced to stretch while it is trying to shorten - a contraction called an eccentric action or muscle lengthening action. Of the three contraction modes, only eccentric actions produce moderate to high degree of EIMD with isometric and concentric contractions generating little EIMD. Here, I will focus on the EIMD models involving eccentric actions in human studies. Because strength loss is considered one of the most reliable indicators of EIMD, I will compare the models with regard to their ability to produce a loss in force.

Human EIMD models can be divided into two major categories: 1) a single eccentric exercise bout of an isolated muscle group; 2) whole body exercise with an eccentric action component or eccentrically biased exercise (e.g. marathon running, downhill running, backward cycling, etc.).

The first category can be further divided into either isokinetic or isotonic models. Isokinetic EIMD models usually use an isokinetic dynamometer while isotonic EIMD models involve lowering weights (or contracting against forces lengthening) to conduct the eccentric actions. In the isokinetic models, the isokinetic dynamometer enables the subjects to conduct eccentric actions at a constant angular velocity regardless of the applied load. Therefore, if the subjects are motivated to maximally resist the dynamometer, maximal force can be imposed on the muscles. In contrast, in the isotonic models, the external load (e.g. dumbbells and barbells) is imposed throughout the entire eccentric movement regardless of the angular velocity. Within this first category of EIMD model, elbow flexors and knee extensors are by far the most commonly investigated muscle groups. Using different models involving different muscle groups, our laboratory has investigated EIMD for more than 30 years. For example, using a modified preacher curl device which uses maximal force over the entire range of contraction regardless of the speed, several studies from our laboratory observed (125, 127, 146, 150) that the subjects experienced a 48-65% reduction in isometric strength after 24, 50, or 70 repetitions of maximal eccentric contractions of elbow flexors. Similarly, other laboratories also reported (22, 32, 136) a 45-60% loss in isometric strength after 30, 36, or 70 repetitions of maximal eccentric contractions of elbow flexors. By comparison, relatively less strength loss is produced in the studies that used knee

extensors to perform the eccentric actions. For instance, Brown et al. (15) demonstrated only 24% loss and Chen et al. (23) showed a 33% loss in isometric strength after 100 repetitions of eccentric contraction of knee extensors. Taken together, data from our laboratory and other groups demonstrated that a single bout of eccentric exercise can reliably result in moderate or high degrees of strength loss and likely muscle damage, depending on the specific protocols.

Fewer studies used the second category of EIMD model - whole body exercise with eccentric components or bias. Compared with the first category of EIMD model, only low to moderate degree of muscle strength loss was produced after whole body eccentrically-involved exercise. For example, Millet et al. (107) showed a 30% decrease in knee extensor strength after a 65-km ultramarathon run, and Hickner et al. (45) reported only a 7-10% decrement in leg strength after 30 min of downhill running at - 11% grade.

In summary, a single bout of eccentric exercise using isolated muscle groups or whole body eccentrically-biased exercise can be used to produce EIMD. A single bout of eccentric exercise using isolated muscle groups can generate a higher degree of muscle damage than whole body exercise models.

Markers of EIMD in human studies

Many researchers have described EIMD via assessing a wide variety of markers such as prolonged muscle strength loss, muscle soreness, swelling, increased muscle proteins in the blood, myofibrillar disruption within the muscle, etc. In the following

sections, I will present the evidence from the literature regarding the advantages and disadvantages of using these markers to assess EIMD in human studies.

Direct markers of EIMD

EIMD can be directly assessed via needle biopsy muscles using light or electron microscope. This method can visualize EIMD such as myofibrillar disturbance and ultrastructural disruption, particularly Z-line streaming. In a study conducted by Friden et al. (40) in 1981, myofibrillar disturbance and Z-line streaming were observed in the muscle biopsy samples taken 2 d and 7 d after the subjects performed repeated stairs descents. Two years later, the same group reported that focal disruptions were evident at 1 h, 3 d, and 6 d after eccentric cycling (41). Z-line streaming or even Z-line absence was also observed (41). Subsequently, many other studies also showed focal abnormalities after different eccentric exercise modes. For example, Newham et al. (120) demonstrated greater damage in the muscle biopsy samples at 24-48 h than immediately after eccentric stepping-down exercise. Stupka et al. (157) observed focal damage in the muscle biopsy samples taken at 48 h after eccentric exercise. The ultrastructural disruptions were evident even 10 d after damaging exercise (97, 135). Although Z-line disruption is the predominant disturbance reported in EIMD, A-band disturbance (41, 97), I-band disruption (97), extracellular matrix and capillary disturbances (156) have also been reported. Another interesting phenomena is that the focal disruptions are predominantly observed in type II fibers (41), suggesting that type II fibers are more susceptible to EIMD. The biggest problem using muscle biopsies to quantify muscle damage is that this method assumes that a small piece of biopsy sample is representative of the entire exercised muscle, which is obviously questionable. As mentioned before, the structural

disturbances of EIMD is focal, assessing damage in the whole muscle based on the observations in a small piece of biopsy sample can overestimate or underestimate the real degree of muscle damage. In addition, muscle biopsy itself is invasive and thus can result in damage that can be erroneously taken as EIMD. Therefore, researchers have also used indirect markers for EIMD, some of which will be discussed in the following section.

Indirect markers of EIMD

Strength loss

In a review by Warren et al. (168), 50% of the human studies they reviewed measured maximal voluntary contraction torque. Muscle strength has been considered the best indirect marker for EIMD in humans because it is relatively accurate, reliable, indicative of muscle function, is non-intrusive and can be repeatedly measured over time (168). The magnitude and persistence of strength loss is dependent on different exercise models, as what has been discussed in details in a previous section. Typically, muscle strength is reduced immediately after eccentric exercise and lasts generally one to two weeks post-exercise (28, 119). The strength loss immediately after exercise is also indicative of metabolic fatigue because non-damaging exercise also results in strength loss immediately after exercise (35). The prolonged muscle strength loss after eccentric exercise is probably due to the initial mechanical damage during the exercise and additional damage during the inflammatory response after exercise.

Two issues need to be kept in mind when using strength loss as a marker for EIMD. The first is that torque output is joint angle-dependent. Therefore, measuring the torque at the same joint angle is critical to obtain reliable data and make comparison

between different subjects and studies valid. The second is that muscle strength recovery may be also dependent on the type of movement (isometric, concentric, or eccentric) and/or angular velocity of muscle action. Several studies (36, 41, 43) have shown a slower recovery of concentric strength at faster angular velocity compared to the restoration of isometric or concentric strength at slower angular velocity after damaging exercise. The slower muscle strength recovery at the faster velocities may be due to the selective damage of fast twitch muscle fibers during eccentric exercise (41, 83). Thus, measuring both isometric and isokinetic strength at different speed may give us a better idea about the strength loss.

Increased CK activity in the blood

Increased blood CK level is another commonly measured indirect marker for muscle damage that is generally attributed to its leakage out of the muscle cells with damaged membranes. CK response after damaging exercise is dependent on the exercise modes. For example, blood CK activity peaks approximately 12-24 h after downhill running (18). However, CK activity does not peak until 4-5 d after high intensity eccentric exercise (28).

Many studies use blood CK activity as a marker for EIMD because it is relatively cost-efficient and the magnitude of increase is relatively large. However, it has problems as an indicator for EIMD. High and unexplained inter-subject CK variability post-exercise is often observed (26). Blood peak post-exercise CK activity can range from 236 IU/L to 25,244 IU/L in subjects who performed the same eccentric exercise (128). The other issue is the fact that blood CK activity reflects not only the CK released from the

damaged muscle cells, but also the clearance of CK by other tissues. Because of these issues, it is not surprising that blood CK activity does not correlate well with histological evidence of muscle damage (97). Thus, blood CK activity is only a qualitative indirect marker of EIMD and does not represent a good quantitative marker.

Delayed onset muscle soreness

Of all the cited human studies in Warren et al.'s review, muscle soreness was the most commonly (72%) used marker for muscle damage. Typically, muscle soreness begins many hours after exercise and peaks 2-3 d post-exercise (28). Soreness usually is measured via a visual analog scale. In this type of test, the subjects are asked to mark the peak soreness level felt on a scale. Another way to assess muscle soreness is to determine the threshold force applied to a muscle that induces pain.

Muscle soreness is often used because it is non-invasive and easy to measure. One limitation of using soreness as a marker for EIMD is that the measurement of muscle soreness is, by its nature subjective, and thus vulnerable to any factor that could affect subjects' ability to perceive and evaluate the soreness level accurately.

Proposed Mechanisms Underlying EIMD

Time-course of EIMD

It is generally accepted that the processes/changes occurring after a bout of damaging exercise can be divided into two phases: 1) initial phase or primary damage that is believed to be caused by mechanical stress from the eccentric actions; and 2) secondary phase or secondary damage that is associated with an acute inflammatory

response. The detailed proposed mechanisms driving these changes will be discussed in the following sections.

Mechanisms for initial EIMD

Using light or electron microscope, focal sarcomeric abnormalities, especially Z-line disruption, were observed in the muscle biopsy samples taken immediately after eccentric exercise in humans (120). Loss of desmin staining, a cytoskeletal protein that helps to maintain Z-line, was observed in rabbit muscles at 5 min after the initiation of eccentric contractions (86). Dystrophin is a large protein that plays an important role in connecting contractile apparatus to the extracellular matrix to maintain muscle integrity. Loss of dystrophin staining was reported immediately after eccentric contractions in the muscles from rats (73, 89) and mice (70). Some researchers have proposed models to explain these initial EIMD findings. For example, Morgan et al. (110) proposed a “popping sarcomere” model to explain the muscle damage during eccentric contractions. According to this model, sarcomeres lengthen in a non-uniform pattern during eccentric contractions such that some strong sarcomeres pull weaker sarcomeres apart. When the weak sarcomeres are pulled further and overstretched, damage is generated. In 1993, Lieber and Friden (84) conducted a study in which rabbit *tibialis anterior* muscles were exposed to cyclic eccentric actions at different deformation due to stress. They found that the magnitude of muscle damage was closely related to muscle strain, not muscle force. Duan et al. (33) reported an increase in free calcium in muscles of rats after downhill running. Combining these findings and Morgan’s popping sarcomere hypothesis, Lieber and Friden (85) put forth a model to explain the eccentric-exercise induced initial

mechanical damages. They proposed that the excessive strain induced sarcomeric disruption via popping sarcomere mechanism. Afterwards, disrupted sarcomeres result in increased intracellular calcium concentration. Calcium can then activate proteolytic enzymes such as calpains, which in turn can cleave many cytoskeletal proteins, including desmin and dystrophin. As a result, Z-lines and sarcolemma are disrupted and observed under microscope, as reported in aforementioned studies.

Mechanisms for secondary EIMD

Acute inflammatory response after eccentric exercise

Some studies have shown that the initial mechanical damage after eccentric contractions becomes worse over time. Friden et al. (41) found that focal muscle disturbances in the muscle biopsy samples was evident at 1 h, 3 d and 6 d after the subjects performed an eccentric cycling exercise with maximal degree of damage at 3 d post-exercise. Similarly, Newham et al. (120) observed greater damage in the human muscle biopsy samples taken 1-2 d after eccentric exercise than those taken immediately after exercise. In addition, muscle soreness peaks 24-48 h post-exercise (28, 121). These data suggest that additional/secondary muscle damage occurred after initial mechanical disruptions.

Although the molecular and cellular mechanisms underlying secondary EIMD are not completely understood, the acute inflammatory response after strenuous, eccentric exercise has been suggested to contribute to secondary muscle damage and delay the regenerative processes (160). As the initial step for the inflammatory response, neutrophils, major players in the acute inflammatory response, are recruited from the

circulation into the damaged muscle areas via chemotaxis. Skeletal muscle cells after mechanical strain or injury induces skeletal muscle to release neutrophil chemoattractants (162). The infiltration of neutrophils can be observed as early as 45 min (37) after exercise and persist for up to 24 h (92). Neutrophils release reactive oxygen and nitrogen species (RONS) and pro-inflammatory cytokines such as interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) (19, 123, 124). The neutrophil-generated RONS and pro-inflammatory cytokines initiate the process to clear debris of damaged tissue and thus prepare for muscle regeneration (27). However, excessive generation of RONS and pro-inflammatory cytokines after strenuous, overexertion exercise has been suggested to contribute to secondary muscle damage and delay the regenerative processes (145). Many unknown molecular and cellular signaling pathways may be involved in the secondary EIMD. Here, I will focus on a key regulator of inflammation - Nuclear Factor-kappa B (NF- κ B) signaling pathway.

NF- κ B activation pathway; secondary damage

NF- κ B signaling pathway is a key inflammatory process in response to eccentric exercise (24, 74). Our laboratory recently, and for the first time, provided direct evidence of NF- κ B activation in muscle at 3 h after eccentric exercise in humans (57). NF- κ B represents a family of transcription factors that regulates the expression of over 150 genes and plays a role in many physiological and pathological processes (74). The NF- κ B family consists of five members, RelA (p65), RelB, cRel, p50, and p52, but p65/p50 represent the canonical pathway (113). To be functional, these members must homo- or hetero-dimerize with one another into a NF- κ B complex. Because p65/p50 heterodimer is the predominant form of NF- κ B in skeletal muscle (60), this review will focus on

p65/p50, specifically p65. In the basal physiological condition, NF- κ B is localized in the cytoplasm in an inactive state, binding to the inhibitory proteins known as I κ Bs (113). I κ B family is composed of several I κ B proteins, of which I κ B α and I κ B β are the predominant forms (7). A variety of stimuli, such as RONS and cytokines released by invading neutrophils after eccentric exercise, can activate the IKK complex by phosphorylation (7). IKK complex consists of two kinases (IKK α and IKK β) and a regulatory IKK γ protein (113). The activated IKK complex can then phosphorylate I κ Bs (63). I κ B α and I κ B β are the two main substrates of IKK (7). Phosphorylation of I κ Bs results in the ubiquitination and subsequent proteasomal degradation of I κ Bs. This frees NF- κ B from the inhibition of I κ Bs, and NF- κ B then translocates into nucleus where it binds to the recognition sequence in the target genes and thus regulates their gene transcription level (7). Many of the genes that are regulated by NF- κ B code for pro-inflammatory proteins such as COX-2, MCP-1, and IL-6 (137), thereby increasing inflammation, which is believed to play a major role in the secondary muscle damage after strenuous eccentric exercise (160). This NF- κ B signaling pathway is illustrated in Figure 2.1.

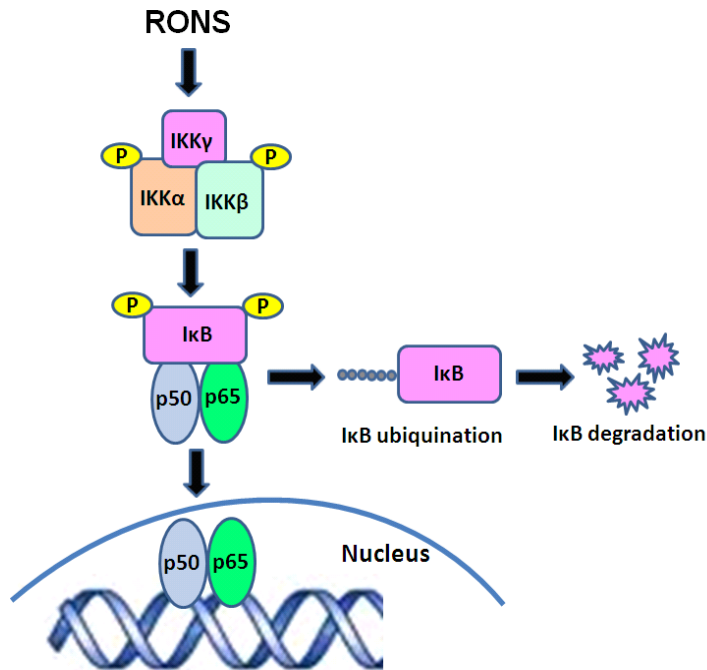


Figure 2.1. Activation of NF- κ B signaling pathway. The NF- κ B signaling pathway is illustrated using Reactive Oxygen and Nitrogen Species (RONS) as a stimulus. See the text for detailed explanation.

Repeated Bout Effect (RBE)

A single bout of strenuous, unaccustomed eccentrically-biased exercise produces muscle adaptation such that the same muscle is less susceptible to EIMD during a subsequent, similar bout of eccentric exercise (55, 126). This phenomenon of less damage after a second bout using the same muscle is referred to as the “repeated bout effect” (RBE) (27, 126). Numerous studies have reported the RBE both in animal (17, 98, 99, 147, 151) and human models (11, 29, 46, 66, 115, 132, 133, 141, 142, 154).

Characteristics of RBE

The repeated bout of effect (RBE) has been reported to last several weeks and up to 6 months after the initial exercise bout (132). However, the magnitude of RBE is

dependent on many factors such as time intervals between bouts, the number of eccentric contractions, and exercise intensity. In 1991, Nosaka et al. (129) investigated the RBE in two groups of subjects who performed two bouts of 70 maximal eccentric actions spaced six weeks and 10 weeks apart. They found that RBE existed in both groups with greater magnitude in the six-week group than the 10-week group. This suggested that RBE decreases as the time interval between bouts increases. It also appears that the initial bout does not need to be the same volume as the second bout to confer a RBE. Clarkson and Tremblay (29) reported that an initial bout of 24 maximal eccentric actions of elbow flexors reduced the magnitude of blood CK activity, strength loss, and muscle soreness when a bout of 70 maximal actions of the same muscle group was performed two weeks later. Similarly, Brown et al. (16) demonstrated that an initial bout of 10, 30, or 50 eccentric actions of knee extensors showed equal protection for a subsequent (three weeks later) bout of 50 eccentric actions of the same leg. A more recent study (133) showed that six or even two maximal eccentric action of elbow flexors conferred a similar protective effect on a second bout of 24 eccentric actions of the same muscle group performed two weeks later. However, eight-week concentric or eccentric training of elbow flexors using a submaximal (50% of maximal isometric force) dumbbell weight have been shown to provide no protective effect on a subsequent (five weeks later) maximal eccentric exercise bout (130). Whitehead (170) et al. even showed that concentric exercise training (450 plantarflexion movements per day for five days) increases the susceptibility of muscle to eccentric exercise-induced damage. Data from these studies (130, 170) suggested that, to confer a RBE, a muscle needs to be stimulated

by exercise with similar intensity and muscle actions specificity (but not number of actions) as those in the damaging exercise.

Proposed mechanisms underlying RBE

Although RBE is widely and well documented, the mechanism underlying this phenomenon is still not fully elucidated. In a review by McHugh (100), the potential mechanisms for RBE were categorized as neural, mechanical, and cellular adaptations.

Neural adaptation

Compared to concentric contractions, eccentric actions require less motor unit activation for a given muscle force (111), and predominantly recruit fast twitch motor units (105). As a result, higher stress is placed on an active fiber and more fast-twitch muscle fibers are involved in eccentric actions. High stress on a small number of active fibers has been postulated as a mechanism for eccentric action-induced muscle damage (111). Because fast twitch fibers have been shown to be more susceptible to eccentric contraction-induced damage, preferential involvement of fast-twitch fibers can partly explain why eccentric contractions are damaging. Thus, it is reasonable to propose that neural adaptations for RBE would include increased amplitude of motor unit activation and increased number of slow-twitch muscle fibers. Indeed, some studies have provided evidence supporting neural adaptation theory. Using surface electromyography (EMG), Hortobagyi et al. (49, 50) showed that EMG activity was increased after eccentric strength training. Warren et al. (167) reported a 30% decrease in median frequency EMG signal during a second bout of eccentric contractions in humans, indicating a shift from fast-twitch to slow-twitch motor units. However, evidence against neural adaptations for

the RBE also exists. A human study (131) has demonstrated that the RBE still occurred when two bouts of eccentric contractions were applied via electrical stimulation. These findings suggested that the RBE was not due fully to neural adaptation because neural activation was bypassed during the eccentric contractions in both studies.

Mechanical adaptation

The proposed mechanical adaptations for the RBE include remodeling of intermediate filament, such as desmin, and increased connective tissue. Barash et al. (10) found that desmin content was increased in rat tibialis anterior muscles 72-168 h after a bout of 30 eccentric contractions. The authors proposed that this increased desmin can provide mechanical reinforcement, which could potentially make the muscle more resistant to sarcomere strain. Lapier et al. (77) observed that increased rat intramuscular connective tissue concentration via limb immobilization was accompanied by attenuation of muscle damage. The authors hypothesized that increased passive stiffness secondary to increased connective tissue after an initial bout of eccentric exercise may dissipate myofibrillar stress during the second exercise bout. Contradictory to mechanical adaptation theory, Sam et al. (148) found that desmin knockout mouse muscles are less vulnerable to eccentric contraction-induced damage compared with wild-type muscles. Also, McHugh et al. (102) demonstrated that subjects with stiffer hamstring muscles experienced greater symptoms of muscle damage, which is opposite to what would be expected to see according to mechanical adaptation theory.

Cellular adaptation

Addition of sarcomeres longitudinally

In 1990, Morgan (110) proposed that addition of longitudinal sarcomeres might be a mechanism for RBE. He hypothesized that the number of longitudinal sarcomeres is increased after an initial bout of eccentric exercise so that less strain will be placed on a sarcomere during the second exercise bout, thus reducing myofibrillar disruption. In support of this theory, Lynn and Morgan (91) reported an increase in serial sarcomeres in rat vastus intermedius muscle fibers after downhill running. In addition, two human studies (14, 104) demonstrated a rightward shift in the length-tension curve after a bout of eccentric contractions, indirect evidence of addition of sarcomeres. However, this shift has been reported to return to normal before the recovery period is complete (104, 171), while RBE showed an accelerated recovery.

Attenuated inflammatory response

As mentioned before, an excessive inflammatory response after eccentric exercise is believed to play a role in eccentric contraction-induced muscle damage (127, 143, 153). Therefore, it is plausible that attenuation of inflammatory response may be a mechanism for RBE. In fact, Pizza et al. (142) reported a reduction in CD11b, CD64, and CD18 on circulating neutrophils and monocytes after a repeated bout of eccentric exercise of elbow flexors. CD11b, CD64, and CD18 are leukocyte receptors and considered as indicators of neutrophil and monocyte activation. The reduction in these leukocyte receptors concurred with a smaller increase in blood CK activity and lower muscle soreness levels after the second bout. In a subsequent study, the same group showed that blood neutrophil numbers were significantly lower at 3, 6, 9 h post-exercise after a second bout of eccentric exercise of elbow flexors (141). In addition, Smith et al.

(154) observed a reduction in serum pro-inflammatory IL-6 and MCP-1 and an elevation in anti-inflammatory IL-10 during the initial 12 h period after the second bout of downhill running compared to the first bout. These findings provided evidence that attenuation of inflammation appears to be a mechanism for RBE. In contrast, Stupka et al. (158) found that the numbers of neutrophils in the quadriceps of women were increased 24 h post-exercise after the second bout of eccentric exercise, while no change was observed in men. The sampling time (24 h post-exercise) may be the reason for the discrepancy between the findings of Stupka et al. (158) and those of other groups mentioned above.

Adaptation in excitation-contraction coupling (E-C coupling)

Ingalls et al. (59) estimated that E-C coupling failure can explain 57-75% of the strength decrement in mouse extensor *digitorum longus* muscles in the first five days after a bout of eccentric contractions. Thus, strengthening of E-C coupling may reduce muscle strength loss after a repeated bout. However, no direct evidence has been reported. However, Lynch et al. (90) found that the intracellular calcium concentration was not different between the mice after a bout of downhill treadmill running and the mice that had been pre-trained with 10 bouts of downhill running.

Summary

In summary, several theories including neural, mechanical, and cellular adaptations have been proposed to explain RBE. However, none of these theories has been entirely verified, with both supporting and refuting evidence existing for each

theory. Most probably, RBE may be a combining effect of some, or all of these potential adaptations.

The Contralateral RBE (CRBE)

The term RBE has been used to mean that the reduced muscle damage after the second damaging exercise bout was observed in the same muscle that has been pre-conditioned with a similar eccentric exercise bout. However, in the past several years, some evidence suggested that the RBE can be transferred to the contralateral muscle groups. Therefore, this section will focus on the CRBE.

There is a well-known cross-transfer effect where an increase in strength in the contralateral (untrained) muscle group has been found following training of only the ipsilateral muscle group (51, 114, 152). However, to our knowledge, only three studies have investigated if RBE also exists in the contralateral muscle group after a single bout of eccentric exercise (30, 54, 155). In two of the three studies, subjects were assigned into one of two groups, an ipsilateral and a contralateral group. Subjects in the ipsilateral group performed the second bout of eccentric exercise with the same arm (elbow flexors) two weeks later; subjects in the contralateral group performed two bouts using the opposite arm in the second bout. Both studies found a significant reduction in muscle strength loss following the second exercise bout for both ipsilateral and contralateral groups, although the magnitude of change was less in the contralateral muscle compared to the ipsilateral muscle (54, 155). Since both studies investigated arms (elbow flexors) it would be important to know if the CRBE also exists in other muscle groups such as in legs. The third study (30), which actually predates the others, had investigated if a single bout of eccentric exercise in one leg can provide protective effect from muscle damage

when a similar bout of exercise performed on the contralateral leg. In that study, subjects performed two bouts of eccentric stepping exercise of quadriceps, with one leg in the initial bout and the other leg in the second bout two weeks later. A significant reduction in muscle pain was found after bout 2 versus bout 1. However, isometric strength loss was not different between two bouts. Given that muscle strength loss is the best indirect marker of exercise-induced muscle damage (168), the authors concluded that the CRBE was not supported and the pain reduction after the second bout was probably because the subjects were more familiar with the discomfort of the testing (30). This conclusion should be taken with caution because muscle damage stimulus in the study was small (muscle torque reduction after bout 1 was only 10% compared with 25% and 30% in the two studies described above) and thus might be insufficient to induce a significant adaptation. Indeed, the authors mentioned that further research using exercise mode with higher damaging effect are warranted to investigate the existence of the CRBE.

Given the paucity of evidence for the existence of the CRBE, it is not surprising that the mechanisms underlying the CRBE are poorly understood. Using EMG analysis to examine the CRBE, Starbuck and Eston (155) concluded that differences in motor unit recruitment due to central adaptation were transferred to the contralateral muscle group. Thus, the decrease in strength loss after a second bout of exercise is likely due to central mechanisms whereby motor unit recruitment is optimized during the second exercise bout leading to less initial damage and thus less strength loss post-exercise. However, the molecular and cellular mechanisms underlying contralateral EBE are still not known. Since NF- κ B plays an important role in the excessive detrimental inflammatory response

after eccentric exercise, it is reasonable to hypothesize that attenuation of inflammatory response involving NF- κ B activation may be involved in the CRBE.

Summary

Unaccustomed, especially eccentrically-biased exercise often results in temporary muscle damage as evidenced by histological myofibrillar disturbances, delayed onset muscle soreness, and prolonged strength loss (27). An acute inflammatory response after the damaging exercise is believed to play a role in EIMD. NF- κ B is a key simulator of inflammatory response after eccentric exercise. Symptoms and signs of EIMD are reduced following a repeated bout of eccentric exercise. To explain this repeated bout effect (RBE), neural, mechanical, and cellular adaptations have been proposed. To date, only a couple of studies have demonstrated the existence of the CRBE. Furthermore, little is known regarding the molecular and cellular mechanisms underlying the CRBE. Therefore, studies are warranted to uncover the mechanisms of the CRBE from transcriptional (e.g. mRNA), post-transcriptional (e.g. microRNA), translational (e.g. protein expression level), post-translational (e.g. protein phosphorylation), and cellular (e.g. neutrophil infiltration) perspectives.

CHAPTER 3

STUDY I

A CONTRALATERAL REPEATED BOUT EFFECT ATTENUATES INDUCTION OF NF- κ B DNA-BINDING FOLLOWING ECCENTRIC EXERCISE

(Adapted from publication in the J Appl Physiol. 116(11):1473-80, 2014)

Abstract

I investigated the existence of the CRBE and tested if the attenuation of nuclear factor-kappa B (NF- κ B; an important regulator of muscle inflammation) induction following eccentric exercise is a potential mechanism. Thirty-one healthy men performed two bouts of knee extension eccentric exercise, initially with one leg, and then with the opposite leg five weeks later. *Vastus lateralis* muscle biopsies of both exercised and control legs were taken 3 h post-exercise. Knee extension isometric and isokinetic strength (60°/sec and 180°/sec) were measured at baseline, pre-exercise, immediately post-exercise, and once/day for five days post-exercise. Serum creatine kinase (CK) activity and muscle soreness were assessed at baseline and once/day for five days post-exercise. NF- κ B (p65) DNA-binding activity was measured in the muscle biopsies. Isometric strength loss was lower in bout 2 than in bout 1 at 24 h, 72 h, and 96 h post-exercise ($P < 0.05$). Isokinetic strength (60°/sec and 180°/sec) was reduced less in bout 2 than in bout 1 at 72 h post-exercise ($P < 0.01$). There were no significant differences between bouts for post-exercise CK activity or muscle soreness. p65 DNA-binding activity was increased following eccentric exercise (as compared to the control leg) in bout 1 ($122.9\% \pm 2.6\%$; $P < 0.001$) and bout 2 ($109.1\% \pm 3.0\%$; $P < 0.05$). Compared

with bout 1, the increase in NF- κ B DNA-binding activity post-exercise was attenuated after bout 2 ($P = 0.0008$). Repeated eccentric exercise results in a CRBE, which could be due to the attenuated increase in NF- κ B activity post-exercise.

Introduction

Unaccustomed exercises, especially those involving eccentric (muscle lengthening) actions, induce transient muscle damage (27, 57, 134). Exercise-induced muscle damage is typically manifested by cellular damage, muscle soreness, prolonged strength loss, and increased blood levels of intramuscular proteins such as creatine kinase (CK) and myoglobin (27). This exercise causes initial mechanical damage to the muscle followed by an acute inflammatory response (160) leading to secondary damage. As part of the inflammatory response, neutrophils are quickly recruited from the circulation into the damaged muscle areas (139). Neutrophils release reactive oxygen and nitrogen species and pro-inflammatory cytokines (19, 24), which then activate transcription factors such as nuclear factor-kappa B (NF- κ B) by a series of events (74). As a result, NF- κ B binds to specific genomic regulatory regions and drives the expression of target gene products, many of which are pro-inflammatory proteins such as cyclooxygenase-2 (COX-2), monocyte chemoattractant protein-1 (MCP-1), and interleukin-6 (IL-6) (137). These proteins increase inflammation that is believed to play a primary role in the secondary muscle damage after strenuous eccentric exercise (24).

Muscle damaging exercise typically results in an adaptation response. For example, a repeated bout of eccentrically-biased exercise produces less damage in a muscle that has been exposed to a similar exercise bout less than six months prior, a

phenomenon known as the “repeated bout effect” (RBE) (27, 126). One of the proposed mechanisms for RBE is attenuated inflammatory response post-exercise (100). In support of this potential mechanism, Pizza et al. (142) reported a reduction in some blood leukocyte receptors after a repeated bout of eccentric exercise of elbow flexors. In a later study, the same group demonstrated that blood neutrophil numbers were significantly lower at 3, 6, 9 h post-exercise after a second bout of eccentric exercise of elbow flexors (141). In addition, Smith et al. (154) observed a reduction in IL-6 and MCP-1 and an elevation in anti-inflammatory IL-10 during the initial 12 h period after the second bout of downhill running compared to the first bout.

To our knowledge, only three studies have sought to determine if the RBE also exists in the contralateral muscle group after a single bout of eccentric exercise (30, 54, 155). In two of the three studies, subjects were assigned to either an ipsilateral or a contralateral group. Subjects in the ipsilateral group performed the second bout of eccentric exercise with the same arm (elbow flexors) two weeks later; subjects in the contralateral group performed two bouts using the opposite arm in the second bout. Both studies observed significant reductions in strength loss following the second exercise bout for both ipsilateral and contralateral groups, although the magnitude of change was lower in the contralateral muscle compared to the ipsilateral muscle (54). The third study (30) reported no evidence of the CRBE in leg muscles. However, the amount of strength loss induced by the first bout in the third study was only 10% compared with 25% and 30% in the two studies described above. Therefore, it is still uncertain if a CRBE also exists in leg muscles because the failure to observe a CRBE in the third study may be due to the insufficient muscle damage stimulus.

Given the paucity of studies exploring the CRBE, it is not surprising that the mechanisms underlying this process have not yet been elucidated. Using electromyography (EMG) analysis, Starbuck and Eston (155) concluded that the observed CRBE is due to neural adaptation whereby motor unit recruitment is optimized during the second exercise bout leading to less initial damage. EMG is usually used to investigate the motor unit activation in the muscle during the actual exercise bout and thus is primarily associated with the initial muscle damage. Therefore, neural adaptation can primarily explain the reduced initial muscle damage during the repeated exercise bout. Since the CRBE includes a reduction in the anticipated muscle damage symptoms up to 10 days or even longer after the exercise bout, other mechanisms are likely to be involved.

Our laboratory recently provided direct evidence of an increase in the activation of the transcription factor NF- κ B (1.6-fold change) at 3 h after eccentric exercise in humans (57). Since NF- κ B plays an important role in the secondary damage response after eccentric exercise (24, 74), I hypothesized that attenuation of NF- κ B activation may be involved in the CRBE. To test this hypothesis, the present study examined muscle function, blood CK activity, muscle soreness, and NF- κ B DNA-binding activity after the initial bout with the ipsilateral leg and after the second bout five weeks later with the contralateral leg. The goals of this study were to determine if the CRBE exists in leg muscles and to identify possible molecular mechanisms for observed effects. I hypothesized that the CRBE also exists in leg muscles and NF- κ B activity would be attenuated in the contralateral leg after the second exercise bout.

Methods

Study Design: The study period consisted of 15 testing visits during which subjects performed two bouts of eccentric exercise spaced five weeks apart (Figure 3.1). In bout 1, subjects exercised one leg (knee extensors); in bout 2, subjects repeated the exercise bout with the knee extensors of the contralateral leg. This study was originally intended to examine the effects of two botanical supplements with anti-inflammatory and antioxidant properties on the muscle response to strenuous exercise. Subjects were randomly assigned in a double-blind manner to receive a formula containing the placebo (inert excipients and processing aids used for supplements) or one of two botanical supplements (supplement 1: rhodiola + rose hips + astaxanthin; supplement 2: ashwagandha + grape seed + prickly pear) for 35 days. However, repeated measures analysis of variance (ANOVA) detected no significant differences between the supplement and placebo groups for CK, muscle soreness or strength ($P > 0.05$). Therefore, I pooled the data from all subjects to further examine the CRBE.

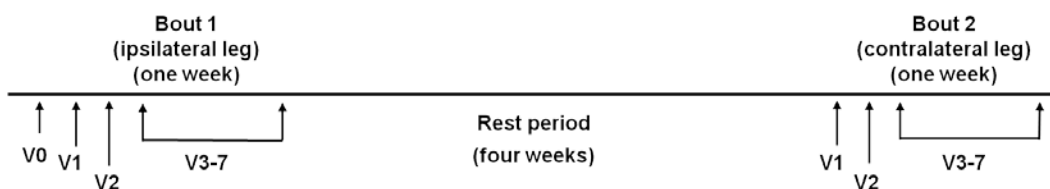


Figure 3.1. Study design. Visit 0 (V0): informed consent and interview; Visit 1 (V1): blood draw, muscle strength tests; Visit 2 (V2): muscle soreness evaluation, pre-exercise muscle strength tests, eccentric exercise, post-exercise muscle strength tests, muscle biopsies 3 h post-exercise; Visits 3-7 (V3-7): blood draw, muscle soreness evaluation, muscle strength tests.

Subjects: Thirty-one healthy men (age = 20.7 ± 0.5 yr, height = 178.5 ± 1.3 cm, Weight = 81.1 ± 3.1 kg; mean \pm SD) completed the study. All subjects were recruited from the local community and signed the informed consent form approved by the Institutional Review Board (IRB) of the University of Massachusetts Amherst. Subjects were sedentary using the standard activity level of less than six metabolic equivalent tasks (METs) and they had not participated in resistance training of the legs within the previous six months. Subjects were excluded if they were unwilling to refrain from taking dietary supplements or non-steroidal anti-inflammatory drugs during the course of the study (except the botanical supplements or the placebo) and if they were smokers. No subjects had skeletal, muscular or neuromuscular dysfunction, or any other known medical conditions that could prevent them from completing the study exercise requirements.

Study Visits: The study design is illustrated in Fig. 1. On Visit 0, subjects gave written informed consent. There were seven visits for both bout 1 and bout 2 periods. The leg tested (exercised) in bout 1 was determined by alternating from one leg to the other as subjects were recruited. Therefore, there were an equal number of right and left legs tested (exercised) in bout 1. Since leg dominance is less profound than upper limb dominance, the approach to balance left and right leg should have provided a balanced design. During Visit 1, subjects had baseline muscle strength tests on the randomized leg and a fasting (> 8 h) blood draw for CK analysis. Visit 2 occurred 24 h after Visit 1; subjects came to the lab after an overnight fast (~ 12 h). Upon arrival for Visit 2, subjects consumed a standardized breakfast of about 0.4 kcals (approximately 55% carbohydrate, 30% fat, and 15% protein). Subjects were then administered a baseline muscle soreness

evaluation and a pre-exercise strength measure, then exercised one leg (knee extensors) followed immediately by a post-exercise strength measure. A muscle biopsy of both eccentric exercised (ECC) and control (CON) legs (vastus lateralis muscle) was taken at 3 h post-exercise. On each day of the following five days (Visits 3-7), subjects were assessed for soreness, strength, and serum CK. During the bout 2 period, subjects repeated the regimen of Bout 1 except that the contralateral knee extensors were exercised.

Muscle Strength: Measures of isometric and isokinetic strength of the knee extensor muscles were assessed on the Biodex System 4 isokinetic dynamometer (Biodex Medical Systems, Shirley, NY, USA). Subjects were tested in the seated position with the lateral femoral epicondyle aligned with the axis of rotation of the dynamometer. Full knee extension (0°) was entered as a reference value into the computerized dynamometer system. Three (3 sec) trials at 70° of knee flexion with one minute rest between trials were assessed for isometric strength. Isometric peak torque values were defined as the average of the highest obtainable value among the three trials. Since several studies (36, 41, 43) have shown a slower recovery of concentric strength at faster angular velocity compared to the restoration of isometric or concentric strength at slower angular velocity after damaging exercise, we measured both isometric and isokinetic strength at different speeds for a more comprehensive analysis of strength loss. Therefore, after a 5-min rest post isometric strength test, subjects performed three consecutive isokinetic strength measures at speeds of $60^\circ/\text{sec}$ and $180^\circ/\text{sec}$ with two minutes rest between each set of three repetitions. Isokinetic extension peak torque values were defined as the average of

the highest value among the three trials. The intra-assay coefficient of variation (CV) of isometric, isokinetic strength at 60°/sec and 180°/sec was 4.2%, 3.1%, and 4.9%, respectively, suggesting the repeatability of the strength measures.

Creatine Kinase (CK) activity: Blood collected from the antecubital vein was allowed to clot for ~15 min. Serum was then obtained by centrifugation for 15 min at 4,400 rpm. Serum samples were analyzed for CK activity using standard clinical procedures (Abbott Laboratories, Worcester, MA). The intra-assay CV was 3.9%.

Muscle Soreness: Soreness/pain was evaluated using a visual analog scale (VAS), a 100 mm horizontal line with 0 mm on the left indicating “no pain” and 100 mm on the right indicating “unbearable pain”. After performing two full squats against the subjects’ own body weight, the subject placed a vertical line through the 100 mm line corresponding to the peak level of pain experienced during the squats. The distance from the left end of the scale to the mark was regarded as the soreness level. All subjects provided pre-exercise (baseline) soreness evaluation. Subjects were only cleared to participate if the baseline VAS score was determined to be > 10 mm in each leg.

Eccentric Exercise: Subjects were seated on the dynamometer chair and completed 10 sets of 10 eccentric repetitions at a speed of 30°/sec with 10 sec rest between repetitions and 1 min rest between sets. The start position of the eccentric exercise was 35° of knee flexion. During each eccentric contraction, the subject was verbally encouraged to maximally extend or “kick” his leg against the dynamometer, which moved at 30°/sec from 35° to the subject’s maximal flexion angle in the normal seated position. At the end

of each eccentric contraction, the tester moved subject's leg back to the 35° start position. The work performed during each set was measured and the total work accomplished during the exercise bout was calculated by adding up the work of the 10 sets.

Muscle Biopsy: A percutaneous needle muscle biopsy was obtained from both non-exercised and eccentric- exercised legs at 3 h following the eccentric exercise. Two biopsies on the same leg were performed at least 3 cm apart to minimize the confounding effect from biopsy procedure. The muscle biopsies were performed under local anesthesia using 2% lidocaine. A small incision (about 1-3 cm) was made in the skin and fascia. A Bergstrom 5-6 mm biopsy needle was then inserted into the muscle and a small core of tissue (about 50-200 mg) was removed and snap-frozen in liquid nitrogen. The wound was closed using 2-3 sutures and the leg wrapped in a compression bandage. An ice bag was applied to the biopsy area while the subject rested for about 15 min. The collected tissue was stored at -80°C until analysis.

ELISA-Based NF- κ B (p65) DNA-Binding Activity: Nuclear extract isolation was performed using a protocol that we described previously (57). Briefly, nuclear extracts were prepared by homogenizing muscle biopsy samples (>20 mg) in a low salt lysis buffer. Homogenized tissue was then subjected to two cycles of freeze/thaw using an ethanol/dry ice freeze bath and a 37°C water bath. Samples were then vortexed and centrifuged at 3000 rpms for 3 min at 4°C to separate the supernatant (cytoplasmic extracts) from the nuclear pellet. The nuclear pellet was re-suspended in high salt buffer. The samples were then incubated on ice for 30 min followed by centrifugation at 13,000

rpm for 5 min at 4°C. The supernatant, which contained the nuclear protein fraction, was stored at -80°C. A standard bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA) was used to quantify nuclear proteins. NF-κB DNA-binding activity was determined using nuclear extracts and an ELISA-based TransAM NF-κB p65 assay kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions as describe previously (57). Briefly, 12 µg of nuclear extract was added to wells coated with a consensus binding sequence (5' -GGGACTTTCC- 3') for NF-κB and incubated for 1 h at room temperature. Wells were then washed, and a primary antibody against p65 subunit was added and left to incubate for 1 h. Next, all wells were treated with a secondary antibody conjugated to horseradish peroxidase (HRP). A subsequent colorimetric reaction was initiated with the addition of a developing solution for 5-7 min followed by the application of a stop solution. The absorbance of the plate was then read at 450 nm on a microplate reader (FLUOstar Optima, BMG Labtech, Offenburg, Germany). Wild-type and mutated consensus oligonucleotides were used as competitors for NF-κB binding to ensure specificity of the reaction. All samples were run in duplicate, and the average value was used for data analysis. The intra-assay Coefficient of Variability (CV) was 5.1%.

Data Analyses: A paired t-test was used to analyze the total work during the exercise that the subjects completed in bouts 1 and 2. Using baseline and pre-exercise muscle strength data, Pearson correlation analysis and a paired t-test was performed to assess the test-retest reliability of the muscle strength measurements. Muscle strength, CK, and soreness data were analyzed via a repeated measures ANOVA to obtain the main effects

of time (exercise), bout (bout 1 vs. bout 2), and the interaction term. CK data were not normally distributed and were log transformed prior to performing the ANOVA. The NF- κ B DNA-binding activity data were expressed in arbitrary absorbance units (450 nm) and analyzed via ANOVA to obtain the main effects of exercise (ECC vs. CON), bout (bout 1 vs. bout 2), and the interaction term. When appropriate, Tukey's *post-hoc* analysis was performed. All statistical tests were conducted using a SAS statistical software package (V9.2; SAS Institute, Cary, NC, USA) with significance set at $P < 0.05$.

Results

Total Exercising Range of Motion and Work Completed per Bout: The total exercising range of motion was 76.4 ± 6.5 degree and 78.3 ± 6.8 degree for bout 1 and bout 2, respectively. The exercising range of motion difference (1.9 ± 6.4 degree) between the two bouts was not statistically significant ($P = 0.26$). The amount of total work completed by subjects during the eccentric exercise for bout 1 and bout 2 was 16123.0 ± 4088.9 J and 16528.5 ± 4335.9 J, respectively. The difference in the amount of total work performed between the two bouts was 405.5 ± 2815.7 J and this difference was not statistically significant ($P = 0.44$). The consistency of exercising range of motion and total work performed between bouts is evidence that the data are not significantly confounded by the exercise protocol design.

Muscle Strength: Muscle strength was measured at baseline, pre-exercise (Pre), immediately after exercise (Post), and every 24 h for 120 h following two bouts of eccentric exercise. Table 3.1 displays the baseline and pre-exercise strength data. All

Pearson correlation coefficients (r) of baseline vs. pre-exercise values were highly significant ($P < 0.005$). Paired t-test results indicated that there was no significant difference between baseline and pre-exercise values for isometric strength and isokinetic strength at $60^\circ/\text{sec}$. For isokinetic strength at $180^\circ/\text{sec}$, the out 1 baseline value was significantly lower than other values (bout 1 pre-exercise, bout 2 baseline and pre-exercise values), due perhaps to the familiarization process. Overall, the muscle strength data were judged to be reliable. For all muscle strength variables, the pre-exercise values were included while baseline values were excluded from statistical analysis because the pre-exercise test was closer to the subsequent measurement time points and thus minimizing the confounding effect from the familiarization process.

Table 3.1. Baseline and pre-exercise values of isometric (at 70° of knee flexion) and isokinetic (at $60^\circ/\text{sec}$ and $180^\circ/\text{sec}$) knee extension peak torque (N·m)

| | Baseline | Pre-Exercise | Difference Between Baseline and Pre- Exercise |
|---------------------------------------|--------------------|------------------|---|
| Isometric | | | |
| Bout 1 | 230.0 ± 47.7 | 233.1 ± 58.3 | 3.1 ± 23.6 |
| Bout 2 | 235.3 ± 48.5 | 229.8 ± 44.8 | 5.1 ± 23.2 |
| Isokinetic ($60^\circ/\text{sec}$) | | | |
| Bout 1 | 188.2 ± 44.2 | 197.0 ± 44.0 | 8.9 ± 24.9 |
| Bout 2 | 188.2 ± 37.3 | 187.5 ± 36.9 | 0.4 ± 11.6 |
| Isokinetic ($180^\circ/\text{sec}$) | | | |
| Bout 1 | $124.5 \pm 36.3^*$ | 133.3 ± 37.5 | 8.8 ± 20.3 |
| Bout 2 | 134.1 ± 32.7 | 137.6 ± 31.1 | 4.1 ± 11.7 |

Data are mean \pm SD (N = 30). * Significantly different from bout 1 pre-exercise, bout 2 baseline and pre-exercise values.

Figure 3.2A shows the percent change in isometric peak torque at 70° of knee flexion after two bouts of exercise. There were significant bout ($F_{1,29} = 21.23$, $P < 0.0001$), time ($F_{6,174} = 22.64$, $P < 0.0001$), and interaction ($F_{6,170} = 2.78$, $P = 0.013$) effects. The maximal isometric torque loss was observed at 24 h post-exercise after both exercise bouts, decreasing by $42.1 \pm 4.3\%$ and $28.4 \pm 4.1\%$ in bout 1 and bout 2, respectively. Tukey's *post hoc* tests showed that isometric torque was significantly lower in bout 1 than in bout 2 at 24 h ($P = 0.034$), 72 h ($P = 0.002$), and 96 h ($P = 0.035$) post-exercise. The isometric torque returned to pre-exercise levels at 96 h after exercise in bout 1 and 72 h after bout 2, suggesting a faster force recovery after bout 2. Figure 3.2B illustrates isokinetic knee extension peak torque at 60°/sec. The analysis demonstrated significant bout ($F_{1,29} = 6.56$, $P = 0.016$), time ($F_{6,174} = 37.19$, $P < 0.0001$), and interaction ($F_{6,170} = 3.30$, $P = 0.004$) effects. The isokinetic torque at 60°/sec returned to pre-exercise level at 120 h after exercise bout 1 and 72 h after bout 2, suggesting more rapid force recovery after bout 2. Tukey's *post hoc* tests showed that isokinetic torque at 60°/sec was significantly ($P = 0.006$) less reduced in bout 2 (average 9.4% loss) than bout 1 (average 26.9% loss) at 72 h post-exercise. Data for isokinetic peak torque at 180°/sec are depicted in Figure 3.2C. There were significant bout ($F_{1,29} = 13.36$, $P = 0.001$) and time ($F_{6,174} = 21.98$, $P < 0.0001$) effects, but no significant interaction ($F_{6,170} = 1.77$, $P = 0.108$). Tukey's *post hoc* tests showed that isokinetic torque at 180°/sec was significantly ($P < 0.01$) less reduced in bout 2 (average 8.9% loss) than bout 1 (average 20.9% loss) at 72 h post-exercise.

Serum CK Activity: There was a significant increase in CK activity over time ($F_{5, 145} = 48.58, P < 0.0001$) after both bout 1 and bout 2 (Figure 3.3). There were no significant differences in the CK increase between bout 1 and bout 2 ($F_{1, 29} = 0.73, P = 0.398$) and there was no significant interaction ($F_{5, 138} = 0.88, P = 0.496$). Of the 30 subjects who had complete samples for analysis, three were deemed to be outliers because CK levels increased more than three times the standard deviation (peak values of 5,992 U/L, 14,318 U/L, and 20,078 U/L, respectively). Excluding the outlier values did not change any of the results listed above to significant level.

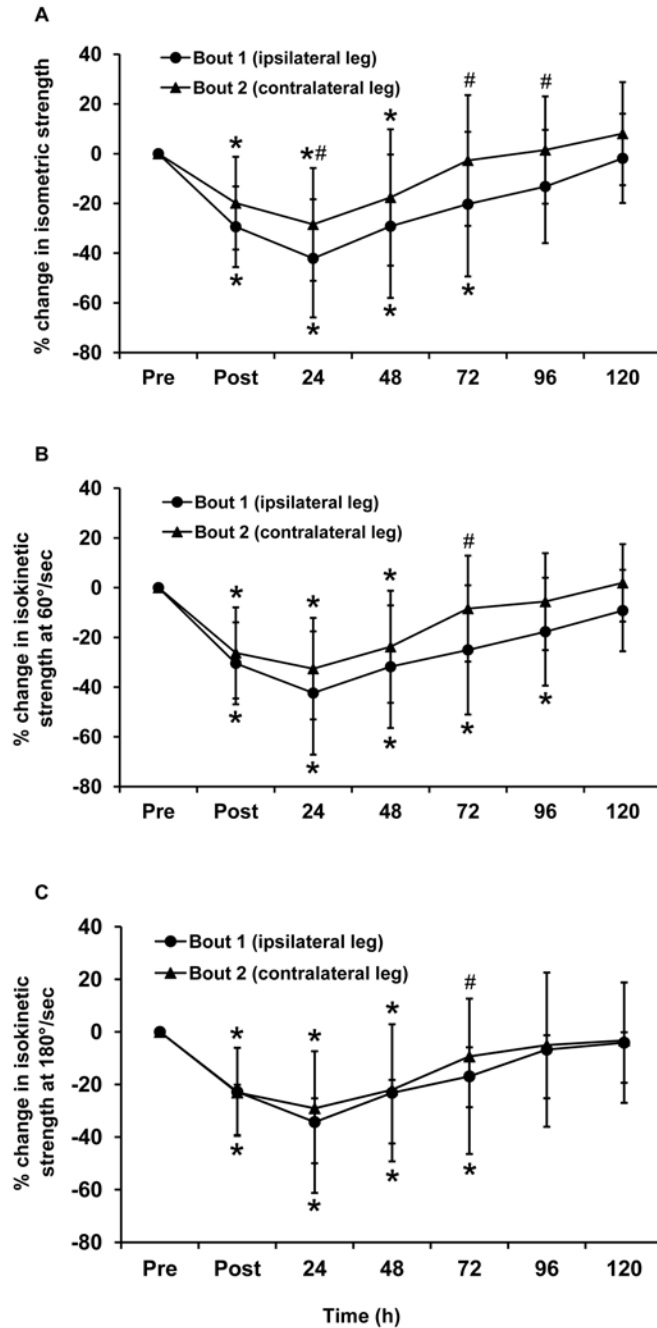


Figure 3.2. Muscle strength changes following two bouts of maximal eccentric exercise in opposite legs; N = 30 for each time point. A. Changes of isometric knee extension peak torque at a joint angle of 70° of knee flexion. B. Changes of isokinetic knee extension peak torque at 60°/sec. C. Changes of isokinetic knee extension peak torque at 180°/sec. Values are presented as mean ± SD. * Significant change compared with pre-exercise values ($P < 0.05$). # Significant change compared with bout 1 ($P < 0.05$).

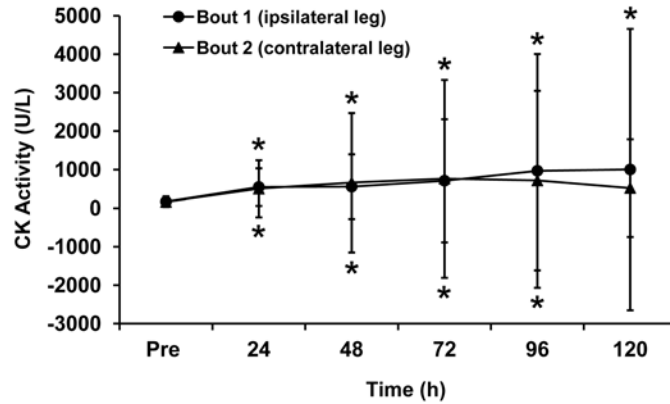


Figure 3.3. Serum CK changes following two bouts of eccentric exercise in opposite legs; N = 30 for each time point. Values are presented as mean \pm SD. * Significant change compared with pre-exercise values ($P < 0.05$).

Muscle Soreness: Muscle soreness was evaluated pre-exercise (Pre) and every 24 h for 120 h following the two bouts of eccentric exercise (Figure 3.4). There was a significant increase in soreness over time ($F_{5, 150} = 88.73$, $P < 0.0001$). Muscle soreness peaked at 24 h after exercise and returned to pre-exercise levels at 96 h post-exercise, independent of bout. There were no significant bout ($F_{1, 30} = 1.13$, $P = 0.296$) or interaction ($F_{5, 144} = 1.06$, $P = 0.387$) effects.

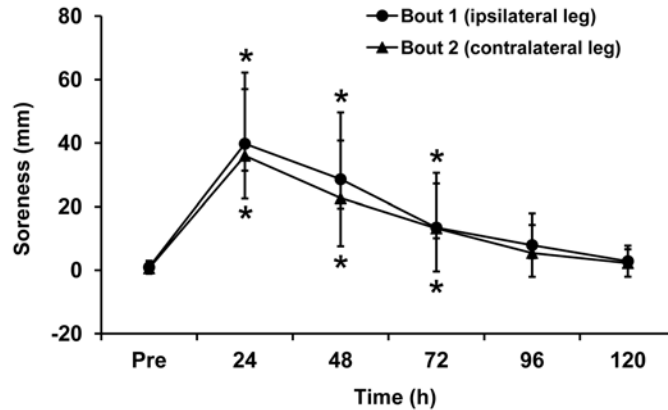


Figure 3.4. Muscle soreness following two bouts of maximal eccentric exercise in opposite legs; N = 31 for each time point. Values are presented as mean \pm SD. *Significant change compared with pre-exercise values ($P < 0.05$).

NF- κ B (p65) DNA-binding Activity: For the majority of subjects there was adequate tissue from the muscle biopsy samples to perform the NF- κ B DNA-binding activity for both bout 1 and bout 2 (N = 26). There were no significant differences between the subcohort and whole cohort (N = 31) for age, height, weight, CK, muscle soreness, or any strength measure at any time point ($P > 0.05$). Figure 3.5 shows the p65 DNA-binding activity level, presented in arbitrary absorbance units (450 nm), after two bouts of exercise. There was significant Exercise ($F_{1,25} = 53.13$, $P < 0.0001$) effect and interaction ($F_{1,25} = 14.38$, $P = 0.0008$), but no significant bout ($F_{1,25} = 1.20$, $P = 0.284$) effect. Tukey's *post hoc* tests demonstrated that p65 DNA-binding activity was increased following eccentric exercise in both bout 1 ($P < 0.001$) and bout 2 ($P = 0.042$). This confirmed our previous finding of NF- κ B activation following eccentric exercise (57). The significant interaction suggested that the increase in NF- κ B DNA-binding activity post-exercise was attenuated in the contralateral leg in bout 2 (ECC relative to CON, $109.1\% \pm 3.0\%$), compared with bout 1 (ECC relative to CON, $122.9\% \pm 2.6\%$).

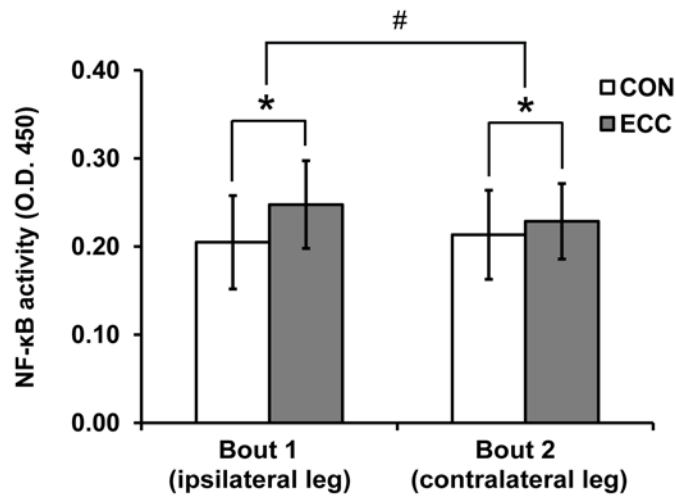


Figure 3.5. NF- κ B (p65) DNA-binding activity measured at 3 h after two bouts of eccentric exercise in opposite legs; N = 26. Values are expressed as mean \pm SD. * Significant change compared with CON leg (P < 0.05). # Significant bout by exercise interaction (P < 0.05).

Discussion

The overall objectives of the current study are to examine the existence of the CRBE in leg muscles and determine the possible involvement of NF- κ B in this phenomenon. Our results demonstrated that there was significantly less muscle strength loss in the contralateral leg after the second exercise bout, suggesting the existence of a CRBE in our exercise model. Furthermore, I found an attenuated increase in NF- κ B DNA-binding activity 3 h after the second exercise bout in the contralateral leg, suggesting that the CRBE is associated with an attenuated NF- κ B activation in muscle following eccentric exercise, which may provide a regulatory mechanism.

The most commonly measured indirect markers for exercise-induced muscle damage are: prolonged muscle strength loss, delayed onset muscle soreness and blood CK levels (27). The eccentric exercise protocol used in this study effectively induced muscle damage as indicated by the significant Time main effect ($P < 0.0001$) for all the measured indirect markers of muscle damage (strength loss, soreness, CK). These findings are in agreement with previous studies (13, 55, 61).

Strength response to the contralateral repeated bout of eccentric exercise

Muscle strength has been considered the best indirect marker for exercise-induced muscle damage because it is relatively accurate, reliable, and indicative of muscle function (168). Because muscle recovery may be also dependent on the type of movement (isometric or isokinetic) and/or angular velocity of muscle action, I measured both isometric and isokinetic strength of the quadriceps at two angular velocities ($60^\circ/\text{sec}$ and $180^\circ/\text{sec}$). For all the muscle strength variables, there was significant lower strength loss and faster strength recovery in the contralateral leg after bout 2. These results suggest that the CRBE exists in leg muscles regarding muscle strength loss in our exercise model.

To date, only two studies (54, 155) reported on the CRBE, both of which were conducted on arm muscles (elbow flexors). Our results support these two studies and extend them to leg muscles. To the best of our knowledge, only one study (30) was conducted with the primary purpose to examine the existence of the CRBE in leg muscles. However, the authors in that study found no significant difference in isometric strength loss after both exercise bouts. The failure of this study (30) to observe the CRBE in leg

muscles may be due to the relatively modest muscle damage stimulus (~10% muscle loss after exercise), which may have been insufficient to induce detectable levels of adaptation. In contrast, there was an approximately 40% loss in muscle strength after Bout 1 in the current study. Although the primary goal was not to examine the CRBE, a study conducted by McHugh and Pasiakos (103) also provided data suggesting that the CRBE is not existent in leg muscles. It should be noted that in McHugh and Pasiakos's study, the exercising range of motion was limited to 40° (either from 30° to 70° or from 70° to 110°), which may have been insufficient to induce a significant CRBE. Furthermore, our data also indicate that the CRBE is evident at least five weeks after the initial bout; previous studies reported that the CRBE was found after two weeks. Taken together, the strength data presented in this study provide additional support for the existence of a CRBE.

CK response to the contralateral repeated bout of eccentric exercise

I observed a similar increase in serum CK after both exercise bouts, suggesting no CRBE for CK. This result does not concur with findings of Howatson and van Someren (54), who reported that the CK increase was attenuated in the contralateral arm 96 h after a second exercise bout. One possible reason why I did not observe a CRBE for CK in the current study may be due to the high inter-subject variability. High inter-subject CK variability post-exercise is observed frequently (26). Indeed, Connolly et al. (30), who were the first group that investigated the CRBE, discarded their CK data from analysis for this reason. In the current study, some subjects had very high post-exercise CK levels (e.g. 20,078 U/L), while others displayed only a relatively modest increase (e.g. 199

U/L). Even after I excluded the data from the three outliers, the standard deviation of CK was still large and represented almost half of the mean values for most time points post-exercise in Bout 1 and even higher in bout 2.

Soreness response to the contralateral repeated bout of eccentric exercise

In the three studies that primarily investigated the CRBE (30, 54), muscle soreness/pain was attenuated in the contralateral limb after the second exercise bout. In contrast, our data failed to demonstrate a significant difference in muscle soreness between bout 1 and bout 2. Similar to the three published studies, I used the visual analog scale (VAS) to evaluate muscle soreness. The peak average muscle soreness after bout 1 observed in our study was close to the magnitude of soreness/pain after Bout 1 in the three previous studies (30, 155). A major difference between the current study and the three earlier reports is that I included muscle biopsies in our study. The measurement of muscle soreness via the VAS is subjective and therefore susceptible to any factor that could affect the subjects' ability to perceive and evaluate the soreness level accurately. The discomfort from the muscle biopsy may be a confounding factor in the measurement of muscle soreness in our study, hindering our ability to detect a RBE. The possible confounding effect of the biopsy is supported by our observation that soreness peaked at 24 h post-exercise, while other studies consistently show that soreness typically peaks at about 48 h post-exercise.

NF- κ B DNA-binding activity response to the contralateral repeated bout of eccentric exercise

I observed that NF- κ B DNA-binding activity was significantly higher in the ECC leg compared to CON leg in both bout 1 and bout 2, suggesting that NF- κ B was activated post-eccentric exercise. The activation of NF- κ B following exercise has been consistently demonstrated in rodent models (47, 63, 87) as well as human studies using peripheral blood lymphocytes (42, 64). However, the literature regarding the effect of exercise on NF- κ B activity in human skeletal muscle is still limited, and the results from different studies are equivocal (34, 57, 159, 166). Therefore, the observed increase of NF- κ B DNA-binding activity post-eccentric exercise in the current study reinforces acute exercise as a stimulus for NF- κ B activation.

An acute inflammatory response after strenuous eccentric exercise has been suggested to contribute to secondary muscle damage and delay the regenerative processes (145). Accumulating evidence suggests that NF- κ B is a critical transcriptional regulator in the acute inflammatory response following exercise (24, 74). NF- κ B is a ubiquitously expressed transcription factor and regulates the expression of about 150 genes, many of which encode pro-inflammatory mediators such as inducible nitric oxide synthase (iNOS), IL-6, IL-8, IL-1 β , MCP-1 (137). Concomitant NF- κ B activation and up-regulation of pro-inflammatory genes following exercise has been reported in both rodent (24, 82, 87) and human studies (57, 64, 166). Since pro-inflammatory cytokines can increase protein degradation (81, 160, 164), it is conceivable that activated NF- κ B expression following strenuous exercise may contribute to secondary muscle damage via up-regulating pro-inflammatory genes.

Given the importance of NF- κ B in the acute inflammatory response that contributes to secondary muscle damage after eccentric exercise, I speculate that the diminished activation of NF- κ B in the contralateral leg in bout 2 may contribute to the observed CRBE in the current study. Although there are no data to demonstrate a cause-effect link between attenuated activation of NF- κ B and the CRBE, there is indirect evidence to support that inhibition of NF- κ B activation is associated with reduced muscle damage. Genetic ablation of NF- κ B activation in mice improved muscle regeneration and limited infiltration of inflammatory cells into damaged muscle (2, 112). Similarly, administration of antioxidant such as Honokiol (24) or melatonin (4) in rats reduced exercise-induced muscle damage paralleled with attenuated NF- κ B activation and down-regulation of pro-inflammatory genes regulated by NF- κ B. Furthermore, six or eight weeks of human eccentric exercise training program attenuated exercise-induced NF- κ B activation and reduced muscle damage (42, 64). Collectively, these data suggest that the attenuated NF- κ B may contribute to less muscle strength loss in the contralateral leg in bout 2.

It should be pointed out that the contralateral leg was not exposed to exercise in the initial bout and thus there was no direct stimulus for molecular and cellular adaptation such as reduced NF- κ B activation in the contralateral leg. Therefore, the attenuated NF- κ B may not be a driving or independent mechanism for the CRBE in the current study. Instead, NF- κ B may be an effector of an upstream mechanistic pathway which could be transferred to the non-exercising contralateral leg muscles. Neural adaptation is most probably the candidate mechanism upstream of NF- κ B because previous studies (52, 155) have demonstrated that neural adaptation is critical for the CRBE. In humans,

performing unilateral lengthening actions of left wrist flexor resulted in an increase in the corticospinal excitability and almost abolished the intracortical and interhemispheric inhibition for the contralateral relaxed right wrist flexor muscle (52). In addition, EMG data (155) showed that there was an increased recruitment of slow twitch motor units in the contralateral muscle during the repeated bout of eccentric exercise. Since fast twitch fibers have been shown to be more susceptible to eccentric contraction-induced damage (41, 65), the shift from fast-twitch to slow-twitch motor units recruitment can result in less initial muscle damage during and immediately after the exercise bout. As a result, a smaller inflammatory response is initiated which may include the attenuated NF- κ B activation. From this perspective, the attenuated NF- κ B activation is just a consequence not the cause of the CRBE. Regardless if attenuated NF- κ B is an independent or effecting mechanism, NF- κ B may be an important mediator or synergist in the observed the CRBE.

There are several limitations to this study and thus cautions should be taken while interpreting findings observed in this study. First, although I did not find significant differences between the supplement and placebo groups for all the measured variables, the supplements may have had a small non-significant confounding effect. Second, I did not recruit an ipsilateral control group to avoid confounding effects of the well-documented ipsilateral RBE while examining the effects of the supplements on muscle recovery post-damaging exercise. Third, the subjects performed the two exercise bouts with five weeks apart so that it is long enough to observe the possible effects of the supplements. However, the CRBE might be more profound if the second exercise bout was performed sooner after the first bout and that might be part of the reason why I did

not detect a CRBE regarding CK or soreness. Finally, muscle biopsy could also be a confounding factor as aforementioned. Future studies can probably recruit a control group of subjects who will only have muscle biopsies without exercising at all.

Summary

The current study demonstrated that the CRBE exists in knee extensors after maximal eccentric exercise and may have implications in clinical and rehabilitation settings. For example, re-mobilization of a weakened or injured limb produces muscle damage (144) that can impede rehabilitation. Consequently, the CRBE in legs can be taken advantage of when designing a rehabilitation plan. If one leg of a patient was immobilized due to an injury or a disease, eccentric exercise of the healthy leg before rehabilitation begins for the immobilized limb may provide protection against exercise-induced muscle damage for the immobilized leg during rehabilitation exercise, thereby facilitating recovery from injury. Moreover, a CRBE must be considered in studies that make use of alternate limbs for the investigation of interventions to reduce exercise-induced muscle damage, and it may be more appropriate to use a between-subjects design rather than a within-subjects design. More importantly, our findings for the first time suggest that NF- κ B may be involved in the CRBE and point to a mechanistic basis for the CRBE. The identified involvement of NF- κ B in the CRBE may be important in the development of interventions to enhance the CRBE when setting up rehabilitation exercise for a unilaterally immobilized limb and in identifying targets of future therapies to facilitate recovery from injury.

CHAPTER 4

STUDY II

TRANSCRIPTOME SIGNATURE OF THE CONTRALATERAL REPEATED BOUT EFFECT (CRBE) IN HUMAN SKELETAL MUSCLE

Abstract

Excessive inflammation contributes to exercise-induced muscle damage (EIMD). I have recently reported that EIMD in one limb resulted in an attenuated EIMD response in the contralateral muscle when it was challenged with a damaging exercise. Muscles experiencing this contralateral repeated bout effect (CRBE) displayed reduced muscle strength loss and a blunted increase in nuclear factor-kappa B (NF- κ B) DNA-binding activity. The current follow-up study was designed to determine, via an unbiased microarray analysis, if the NF- κ B signaling pathway is a critical regulator for the CRBE. Since NF- κ B DNA-binding activity is only one component of NF- κ B signaling pathway, this study also examined the expression of key NF- κ B regulated genes, as well as the phosphorylation of I κ B α (p-I κ B α), an upstream regulator of NF- κ B activity. Thirty-six men performed 100 maximal eccentric actions of the knee extensors using one leg and repeated the exercise bout with the contralateral leg five weeks later. *Vastus lateralis* muscle biopsies were taken from exercised and control legs 3 h after each exercise bout. Agilent whole-genome microarrays were used to examine gene expression changes. The follow-up pathway, function, and network analyses were conducted using ingenuity pathway analysis (IPA) software. qRT-PCR was used to measure mRNA levels of five genes (*CCL2*, *ANKRD1*, *IL1R1*, *CYR61*, and *TGF β 2*) chosen from microarray data analysis, while I κ B α and p-I κ B α were assessed via Western blotting. Microarray data

analysis revealed that 861 transcripts were significantly altered as a result of the CRBE, including 27 NF- κ B related genes. IPA function analysis demonstrated that inflammatory response was one of the top biological functions altered by the CRBE. In addition, network analysis identified NF- κ B as a key signaling pathway affected by the CRBE. qRT-PCR confirmed that the up-regulation of three NF- κ B-related pro-inflammatory genes (*ANKRD1*, *CRY61*, and *IL1R1*) in response to eccentric exercise was attenuated in bout 2 compared to bout 1. These data provide the first documentation of transcriptomic changes in the CRBE and support the hypothesis that an attenuated inflammatory response mediated by NF- κ B contributes to the CRBE.

Key words: contralateral repeated bout effect (CRBE), nuclear factor-kappa B (NF- κ B), gene expression, inflammatory response

Introduction

A single bout of strenuous or unaccustomed eccentrically-biased exercise leads to temporary muscle damage and associated inflammatory responses (160). Excessive inflammation is believed to play a role in the secondary muscle damage observed after eccentric exercise (24, 74). When exposed to a subsequent, similar damaging eccentric exercise, the same muscle is less susceptible to exercise-induced muscle damage (EIMD) (55, 126). This attenuation of damage in that muscle following a second bout of intensive exercise is referred to as “repeated bout effect” (RBE). Although the mechanisms underlying RBE are not completely known, previous studies have implied that RBE is partially attributed to a reduction in exercise-induced inflammatory responses (141, 142, 154).

There are conflicting data regarding the existence of the CRBE, whereby exercising one muscle group induces an adaptive response in the contralateral muscle group which leads to reduced damage when it is subject to the same exercise regimen. For example, data from Connolly et al. (30) suggested that the CRBE is not present in lower limbs, which was further supported by McHugh and Pasiakos (103). In contrast, several later studies demonstrated that CRBE exists in both upper limb muscle (elbow flexors) (54, 122, 155) and lower limb muscle (knee extensors) (48). In support of the existence of the CRBE, we reported recently (173) that EIMD in one leg caused a contralateral repeated bout effect which was manifested by a more modest reduction of strength loss in the contralateral leg after a second exercise bout when compared to the first exercise bout.

Only limited data are available to explain the mechanisms underlying the CRBE. Using surface electromyography (EMG) analysis, Starbuck and Eston (155) concluded that the CRBE is likely due to neural adaptation whereby motor unit recruitment is optimized during the second exercise bout leading to a reduction in initial muscle damage and thus less strength loss post-exercise. However, little is known about the molecular mechanisms that mediate the CRBE. We recently observed a more modest increase in nuclear factor-kappa B (NF- κ B) DNA-binding activity in the contralateral leg after a second exercise bout compared to the first exercise bout (173). NF- κ B is a transcription factor that drives the expression of many pro-inflammatory genes (137). Since excessive inflammation contributes to secondary EIMD (24, 74), a reduction of NF- κ B DNA-binding activity after a second bout could imply that attenuated inflammation responses might contribute to the CRBE.

The NF- κ B family consists of five members: RelA (p65), RelB, cRel, p50, and p52 (113). To form a functional transcriptional regulator, these members must homo- or hetero-dimerize with one another into a NF- κ B complex. In the basal physiological condition, NF- κ B resides in the cytoplasm in an inactive state through its interactions with inhibitory proteins known as I κ Bs (113). There are several I κ B family members, of which I κ B α is the predominant form (7). A variety of stimuli, such as cytokines released by invading neutrophils after eccentric exercise, can activate the I κ B kinase (IKK) complex by phosphorylation (7). Activated IKK complex can then phosphorylate I κ Bs which results in their ubiquitination and subsequent proteasomal degradation (63). This liberates NF- κ B which then translocates into nucleus where it binds to consensus sequence within the promoters of target genes and drives transcription (7). Many of the genes that are regulated by NF- κ B encode for pro-inflammatory proteins such as COX-2, MCP-1, and IL-6 (137), thereby increasing inflammation, which can ultimately result in the secondary muscle damage after strenuous eccentric exercise (160).

Our laboratory previously provided direct evidence of NF- κ B activation at 3 h after eccentric exercise in humans (57). While we have shown that the increase in NF- κ B DNA-binding activity was reduced in the contralateral leg when it was subsequently subjected to a damaging exercise (173), this was only one component of the NF- κ B signaling pathway that we examined. Also it is not clear if the alteration of NF- κ B signaling pathway is actually a key molecular event in the CRBE. Thus, the overall goal of the present study is to determine if there is a concomitant reduction in NF- κ B signaling during the CRBE and if NF- κ B signaling pathway is a critical component for the CRBE. Muscle biopsy samples were collected at 3 h post-exercise after each of two bouts of

eccentric exercise of the knee extensors (the initial bout with the ipsilateral leg and the second bout five weeks later with the contralateral leg). RNA was isolated and used to generate probes for microarray analysis and the changes in gene expression in bout 2 were compared with those observed following the initial bout of exercise. Particular attention was paid to the NF- κ B related genes. Differential gene expression was validated via real-time PCR (qRT-PCR), I also examined the phosphorylation level of I κ B α (an upstream regulator of NF- κ B). The microarray results characterize the changes in expression of the key genes, signaling pathways, and biological functions during the CRBE and identified NF- κ B signaling as an important regulatory pathway in the CRBE. qRT-PCR data confirmed the attenuated induction of three NF- κ B-associated pro-inflammatory genes after the second exercise bout compared to the initial bout of exercise. My data support the hypothesis that inflammatory responses involving NF- κ B are attenuated after bout 2 as demonstrated by reduced expression of NF- κ B associated pro-inflammatory genes.

Methods

Study Design: All muscle biopsy samples for the current study were collected from a study that was originally designed to investigate the effects of two botanical supplements on muscle recovery after damaging exercise. A detailed description of the study design can be found in a previously published paper (173). Briefly, subjects performed two bouts of eccentric exercise of the knee extensors, initially with one leg and then with the contralateral leg later in the second exercise bout. Each exercise bout block comprised seven visits (*visits* 1-7) with 24 h between every two consecutive visits. On *visit* 2 of the

exercise bout 1 block, subjects performed the eccentric exercise with one leg (randomly assigned) and *vastus lateralis* muscle biopsies from both eccentrically- exercised (ECC) and control (CON) legs were taken at 3 h post-exercise. On *visits* 3-7, indirect markers of exercise-induced muscle damage (e.g., muscle soreness, strength, serum creatine kinase activity) were assessed. After a four-week rest, the exercise bout 2 block began and was identical to the exercise bout 1 block except that the contralateral leg was eccentrically exercised. The time interval between the two actual contralateral eccentric exercise bouts was five weeks. Subjects took the placebo or one of the two botanical supplements with antioxidant and anti-inflammatory properties (see components of the supplements in (173)) during the four-week rest period and the exercise bout 2 block, resulting in a total supplementation time of 35 days. Since the supplement had no significant effects on the outcome measures related to muscle damage, data from the different groups were pooled for analysis in the present study.

Subjects: Muscle biopsy samples from thirty-six healthy men (age = 20.9 ± 0.5 yr, height = 178.7 ± 1.2 cm, weight = 80.4 ± 2.9 kg; mean \pm SE) were included in the current study. The subjects' characteristics have been described in detail previously (57, 173). All subjects provided written informed consent, which was approved by the Institutional Review Board (IRB) of the University of Massachusetts Amherst. Subjects were not physically active (physical activity level was less than six metabolic equivalent tasks (METs) per day) and had no resistance training of the legs in the preceding six months. Subjects had no diagnosis of skeletal muscular injuries or other medical conditions and were willing to refrain from participating in new physical activities or taking analgesics (except the botanical supplement or the placebo) during the study period.

Eccentric Exercise: The protocol for the eccentric exercise has been reported in our previously published papers (57, 173). Briefly, subjects performed 10 sets of 10 repetitions of knee extensors on a Biodex System 4 isokinetic dynamometer (Biodex Medical Systems, Shirley, NY, USA). During each eccentric repetition, the subject was verbally instructed to extend his leg maximally against the dynamometer lever as it moved from 35° to the subject's maximal flexion angle at the speed of 30°/sec, with a resultant range of motion at ~77° during each repetition.

Muscle Biopsies: Muscle biopsies of the *vastus lateralis* muscle from both ECC and CON legs at 3 h post-eccentric exercise were conducted under local anesthesia (2% lidocaine) as previously described (57, 173). To avoid confounding effects from the preceding biopsy, the second biopsy on the same leg was at least 3 cm away from the first biopsy. A small incision was made into the skin and fascia and afterwards the biopsy needle was inserted into the muscle. With manual suction, a small grain of muscle tissue (~100 mg) was withdrawn and snap-frozen in liquid nitrogen. Muscle biopsy samples were stored in the -80°C freezer until analysis.

Total RNA Extraction and Microarray Analysis: Total RNA was isolated from homogenized muscle biopsy samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was generated and hybridized to the commercially available Agilent Whole Human Genome Oligo Microarray kit (Agilent, Santa Clara, CA) according to the manufacturer's directions. The Agilent microarray kit covers ~41,000 human genes and

transcripts. The RNA extraction and microarray hybridization was performed at Gene Logic Inc. (Gaithersburg, MD) using the Agilent One-Color gene expression system.

Quantitative Real-Time PCR (qRT-PCR): To validate microarray data, we used qRT-PCR to measure mRNA levels of differentially expressed genes that were identified in the microarray data analysis. The following genes were analyzed: chemokine (C-C) ligand 2 (*CCL2*); ankyrin repeat domain 1 (cardiac muscle) (*ANKRD1*); interleukin 1 receptor, type 1 (*IL1R1*); cysteine-rich, angiogenic inducer, 61 (*CYR6*); and transforming growth factor, beta 2 (*TGFβ2*). Of the 36 subjects, 17 had sufficient mRNA samples from both bouts 1 and 2 for us to perform qRT-PCR. Total RNA (200 ng) from each sample was reverse transcribed into cDNA using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's specifications. cDNA was amplified in triplicate using SsoFastEvaGreen Supermix (Bio-Rad Laboratories Inc, Hercules, CA) on a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories Inc, Hercules, CA). All samples were run in triplicate for each target gene along with "no template" controls. The mean cycle threshold (Ct) values of the triplicate samples were used for data analysis. The PCR conditions were 30 s at 95°C for enzyme activation, which was followed by 40 cycles of three seconds each at 95°C for denaturation and five seconds at 60°C for annealing/extension. At the end of the final cycle of each reaction, a melting curve analysis was run to monitor the EvaGreen fluorescence continuously throughout the temperature range from 65°C to 95°C in 0.5°C increments and a five-second hold at each degree. Observation of a single melt peak from each sample ruled out the presence of primer dimers. Beta-2-microglobulin (B2M) was used as an internal

control because it has been shown previously to not change in expression after eccentric exercise (94). All forward and reverse primers were designed using NCBI gene sequences with the PrimerQuest program (Integrated DNA Technologies, Coralville, IA) (Table 4.1). A standard curve was generated for the forward and reverse primers for all target genes to determine the amplification efficiency. All primers had efficiencies between 95% - 110% and thus appropriate for the relative quantification of amplified mRNA via $\Delta\Delta C_t$ method. Ct values were normalized to B2M to compensate for variations in quantity and real time efficiency. The fold changes were calculated with the $2^{-\Delta\Delta C_t}$ method.

Table 4.1. Primer Sequences for qRT-PCR

| | Forward Primer | Reverse Primer |
|---------------|--------------------------------|---------------------------------|
| <i>CCL2</i> | 5'-TCGCTCAGCCAGATGCAATCAATG-3' | 5'-TGGAAATCCTGAACCCACTTCTGCT-3' |
| <i>ANKRD1</i> | 5'-AAGCGAGAAACAACGAGAGGCAGA-3' | 5'-AGAAACGTAGGCACATCCACAGGT-3' |
| <i>CYR61</i> | 5'-TGAGTGCCGCCTTGTGAAAGAAAC-3' | 5'-TGGGCCGGTATTTCTTCACACTCA-3' |
| <i>TGFB2</i> | 5'-TGCAGAGAGATGTTTGCACCATGC-3' | 5'-TGACAGGCAAGTAGCTGATCCCAA-3' |
| <i>IL1R1</i> | 5'-TTACACAGGGACCACAGTCTGCAA-3' | 5'-TGCAGTTTCTCCTTAGTGGCTGGT-3' |
| <i>B2M</i> | 5'-TGTCTGGGTTTCATCCATCCGACA-3' | 5'-TCACACGGCAGGCATACTCATCTT-3' |

Western Blot Analysis: Phosphorylation status of I κ B α was determined via Western blot analysis using cytosolic extracts from muscle biopsy samples. The cytosolic extracts were isolated using a protocol as previously described (57) and then stored at -80°C. Equal amounts of protein (12 μ g) from each sample were loaded into a 4-15% gradient sodium

dodecyl sulfate (SDS) polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA) and were separated by electrophoresis. Fractionated proteins were then transferred at 4°C (75V for 45 minutes and then 100V for 15 minutes) to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA). The membrane was then blocked with 5% dry milk in TBST for 1 h at room temperature and then incubated overnight at 4°C with the corresponding primary antibody (polyclonal rabbit anti-I κ B α , 1:1000 in 5% BSA/TBST, cat# 9242; monoclonal mouse-anti-p-I κ B α , 1:1000 in 5% milk/TBST, cat#9246; Cell Signaling Technology, Inc. Danvers, MA). The membrane was then incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (Goat Anti-Rabbit IgG (H + L)-HRP Conjugate, 1:2000 in 5% milk/TBST for I κ B α blots, cat# 170-6515; Goat Anti-Mouse IgG (H + L)-HRP Conjugate, 1:4000 in 5% BSA/TBST for p-I κ B α blots, cat#170-6516, Bio-Rad Laboratories, Hercules, CA). The blots were developed using enhanced chemiluminescence (ECL) (cat# 32106, Thermo Fisher Scientific Inc., Rockford, IL). To verify the equal loading amount of protein per lane, the blots were stripped and reprobed with an antibody against GAPDH (rabbit anti-GAPDH, 1:5000 in 5% BSA/TBST, cat# ab9485, Abcam, MA) in combination with anti-rabbit HRP-conjugated secondary antibody (Goat Anti-Rabbit IgG (H + L)-HRP Conjugate, 1:10000 in 5% BSA/TBST, cat# 170-6515, Bio-Rad Laboratories, Hercules, CA). We chose GAPDH as the loading control because GAPDH has been used as a loading control for Western blot analysis in eccentric exercise models since it does not change in response to eccentric exercise (88). Bands from each blot were quantified via densitometry using BioRad ChemiDoc™ XRS+ System with Image Lab™ Software.

Densitometry calculations of protein bands allowed for a comparison of the changes in the relative amount of p-I κ B α protein in the muscle samples.

Data Analysis: *Microarray data analysis.* Probe set means were generated from the PLIER algorithm (typically 6 iterations) in Expression Console (Affymetrix Inc., Santa Clara, CA) and imported into Partek Genomics Suite (V6.5; Partek Incorporated, St. Louis, MO) for statistical analysis. PLIER (“Probe Logarithmic Intensity Error”) is a model-based signal estimator beneficial to multi-array estimations. Normalized data were \log_2 transformed and ANCOVA (analysis of covariance using age and body mass index as covariates) was used to determine differentially expressed ($P(\text{exercise} \times \text{bout}) < 0.05$) genes in the contralateral leg after bout 2. The differentially regulated genes were then imported into Ingenuity Pathway Analysis software V8.7 (IPA, Ingenuity Systems, Redwood City, CA) for pathway, function, and network analyses. IPA is a web-based software and the generation of the significantly regulated canonical pathways, biological functions, and networks was based on connections between the uploaded genes and all other molecules stored in Ingenuity’s Knowledge Base. Ingenuity’s Knowledge Base is a repository of literature-reported biological interactions between gene products. The IPA canonical pathway and biological function analyses was corrected for multiple testing using the Benjamin-Hochberg multiple-test correction (69). The output of network analysis was a set of molecular networks with up to 35 genes within each network. Each network has an IPA score that represents the negative logarithm of the probability of finding the uploaded genes in the network due to random chance. For instance, a score of 2 indicates that there is a 1% probability that the genes are grouped in a network due to

chance. Therefore, a network with a score of higher than 2 has a 99% confidence of not being created by chance.

Non-microarray data analysis. For the data from qRT-PCR and Western blotting, two-way analysis of variance (ANOVA) was used to analyze the main effects of exercise (ECC leg vs. CON leg), bout (bout 1 vs. bout 2), and the interaction term (exercise × bout). The data were analyzed using a SAS statistical software package (V9.2; SAS Institute, Cary, NC). Significance was set at $P < 0.05$.

Results

Global gene Expression and IPA Analysis Implicate inflammatory response and NF- κ B Signaling In the CRBE: One of the major objectives of this study was to identify genes that were differentially expressed in the contralateral leg after the second exercise bout compared to the first exercise bout. There were 861 significantly differentially expressed transcripts using a cut-off P value (exercise × bout) = 0.05. Simple compilation of the top genes showing the greatest magnitude of change after each exercise bout appeared a pattern of change in skeletal muscle gene expression as a result of the CRBE (Table 4.2). For all top up-regulated genes, and the majority of down-regulated genes, the magnitude of change in bout 2 was attenuated (the change being lower than anticipated) compared to that of bout 1.

The differentially expressed transcripts were further analyzed by IPA to help identify the canonical pathways and biological functions that were altered in the CRBE. The top canonical pathways and functions that were regulated are involved in cell death

and survival, stress responses, inflammatory responses, and extracellular matrix structure and function (Table 4.3 and 4.4). Although I screened the differentially expressed genes using a P (exercise \times bout) of < 0.05 , the actual change pattern of each individual gene was complicated. For example, the attenuated induction of a gene in bout 2 vs. bout 1 (all the top up-regulated genes listed in Table 4.2) could represent a real attenuation of expression in bout 2 or the consequence of higher expression of the CON leg in bout 2 (CON 2) compared to the CON leg in bout 1 (CON 1). In the latter case, the difference between CON 1 and CON 2 could be that the up-regulated gene in bout 1 after exercise has not returned to the baseline level by the time bout 2 exercise was performed or the difference between CON 1 and CON 2 exists due to other unknown reasons such as the difference between the two legs before the subject participates in this study or the supplement applied to the subjects may induce the difference. To more deeply look into the change pattern of the genes, I performed sequential steps to further categorize the differentially expressed genes. First, I avoid the confounding effect of the supplements by eliminating those transcripts with a P (group \times bout) < 0.05 , which results in 796 remaining transcripts. Second, I divided the 796 resultant genes into four clusters: cluster A (54 transcripts, up-regulated in bout 1, P (CON 1 vs. CON 2) < 0.05); Cluster B (231 transcripts, up-regulated in bout 1, P (CON 1 vs. CON 2) > 0.05); Cluster C (35 transcripts, down-regulated in bout 1, P (CON 1 vs. CON 2) < 0.05); and Cluster D (476 transcripts, down-regulated in bout 1, P (CON 1 vs. CON 2) > 0.05). Therefore, for the genes of cluster A and C, the different response in bout 2 vs. bout 1 could be due to the difference between CON 1 and CON 2 leg, or a real different response in bout 2, or combination of both. In contrast, for the genes of cluster B and D, the different response

in bout 2 vs. bout 1 is primarily due to a real different response in bout 2 vs. bout 1. Each of the gene clusters were then uploaded to IPA for further analysis. Regardless the different change pattern, the top altered biological functions were similar which include inflammation, cellular growth and proliferation, tissue development (Table 4.5).

Table 4.2. Top regulated genes between bout 1 and bout 2 (P (exercise × bout < 0.05))

| Gene Symbol | Entrez Gene Name | Type | Bout 1 (ECC vs. CON) | | Bout 2 (ECC vs. CON) | | P (exercise × time) |
|----------------------|---|-------------------------------|-------------------------|--------|-------------------------|--------|---------------------------|
| | | | Fold Change | P | Fold Change | P | |
| Top up-regulated | | | | | | | |
| <i>ANKRD1</i> | Ankyrin repeat domain 1 (cardiac muscle) | transcription regulator | 103.4 | <0.001 | 43.7 | <0.001 | 0.012 |
| <i>CYR61</i> | cysteine-rich, angiogenic inducer, 61 | other | 28.1 | <0.001 | 12.3 | <0.001 | 0.007 |
| <i>GEM</i> | GTP binding protein overexpressed in skeletal muscle | enzyme | 27.9 | <0.001 | 13.2 | <0.001 | 0.024 |
| <i>HSPA6</i> | Heat shock 70kDa protein 6 (HSP70B') | enzyme | 17.4 | <0.001 | 5.9 | <0.001 | 0.040 |
| <i>EIF4E</i> | Eukaryotic translation initiation factor 4E | translation regulator | 17.1 | <0.001 | 9.8 | <0.001 | 0.037 |
| <i>FOS</i> | FBJ murine osteosarcoma viral oncogene homolog | transcription regulator | 15.1 | <0.001 | 5.1 | <0.001 | 0.026 |
| <i>KLHL40</i> | Kelch-like family member 40 | other | 15.0 | <0.001 | 9.6 | <0.001 | 0.025 |
| <i>CCL2</i> | Chemokine (C-C motif) ligand 2 | cytokine | 13.1 | <0.001 | 7.5 | <0.001 | 0.041 |
| <i>HSPA1A/HSPA1B</i> | Heat shock 70kDa protein 1A | enzyme | 10.0 | <0.001 | 5.6 | <0.001 | 0.021 |
| <i>HBEGF</i> | Heparin-binding EGF-like growth factor | growth factor | 9.9 | <0.001 | 6.6 | <0.001 | 0.036 |
| <i>ASB5</i> | Ankyrin repeat and SOCS box containing 5 | transcription regulator | 8.4 | <0.001 | 6.2 | <0.001 | 0.040 |
| Top down-regulated | | | | | | | |
| <i>ABCA1</i> | ATP-binding cassette, sub-family A (ABC1), member 1 | transporter | -4.6 | <0.001 | -2.7 | <0.001 | 0.014 |
| <i>COL10A1</i> | Collagen, type X, alpha 1 | other | -3.5 | <0.001 | -2.2 | <0.001 | 0.028 |
| <i>TRIM66</i> | Tripartite motif containing 66 | transcription regulator | -2.8 | <0.001 | -1.7 | 0.037 | 0.030 |
| <i>TAS2R20</i> | Taste receptor, type 2, member 20 | G-protein coupled receptor | -2.7 | <0.001 | -1.4 | 0.313 | 0.028 |
| <i>SIRT4</i> | Sirtuin 4 | enzyme | -2.6 | <0.001 | -1.9 | <0.001 | 0.048 |
| <i>MED14</i> | Mediator complex subunit 14 | transcription regulator | -2.4 | <0.001 | -1.6 | 0.017 | 0.048 |
| <i>ZNF680</i> | Zinc finger protein 680 | other | -2.4 | <0.001 | -1.3 | 0.275 | 0.031 |
| <i>HBS1L</i> | HBS1-like translational GTPase | translation | -2.3 | <0.001 | -1.5 | 0.021 | 0.042 |
| <i>RBM4</i> | RNA binding motif protein 4 | other | -2.3 | <0.001 | -1.7 | <0.001 | 0.033 |
| <i>CNST</i> | Consortin, connexin sorting protein | other | -2.3 | <0.001 | -1.3 | 0.228 | 0.023 |
| <i>FAM117B</i> | Family with sequence similarity 117, member B | other | -1.9 | <0.001 | -3.0 | <0.001 | 0.028 |
| <i>ANPEP</i> | Alanyl (membrane) aminopeptidase | peptidase | -1.5 | 0.004 | -2.4 | <0.001 | 0.015 |
| <i>HMMR</i> | Hyaluronan-mediated motility receptor | transmembrane receptor | -1.3 | 0.123 | -2.2 | <0.001 | 0.025 |
| <i>GIGYF1</i> | GRB10 interacting GYF protein 1 | other | -1.2 | 0.203 | -2.2 | <0.001 | 0.006 |
| <i>KIAA1407</i> | KIAA1407 | other | -1.1 | 0.609 | -2.1 | <0.001 | 0.027 |
| <i>PCYOX1L</i> | Preylcysteine oxidase 1 like | other | -1.3 | 0.025 | -2.1 | <0.001 | 0.034 |
| <i>FAM178B</i> | Family with sequence similarity 178, member B | other | -1.3 | 0.061 | -2.0 | <0.001 | 0.006 |
| <i>SLC29A2</i> | Solute carrier family 29, member 2 | transporter | -1.4 | 0.003 | -2.0 | <0.001 | 0.028 |

Table 4.3. The 10 top canonical pathways associated with transcripts identified as differentially regulated in bout 2 vs. bout 1 (P (exercise \times bout) < 0.05).

| Canonical Pathways | P Value | Genes |
|---|----------|---|
| EIF2 Signaling | 8.22E-07 | <i>RPS3A,RPL36A,RPL39,RPS10,RPS19,RPL30,RPL21,RPL37A,RPL7,EIF4E,RPS12,RPL7A,RPL23A,RPL37,RPS25,RPS15A,RPS5,RPL41,RPL13A</i> |
| Semaphorin Signaling in Neurons | 4.01E-03 | <i>MET,FYN,RND3,RHOB,RHOF,ARHGAP1</i> |
| mTOR Signaling | 7.88E-03 | <i>RND3,RPS3A,RHOB,PPM1L,RPS19,RPS10,RPS15A,RPS25,RHOF,RPS5,EIF4E,RPS12</i> |
| RAR Activation | 1.33E-02 | <i>SDR9C7,NSD1,FOS,SRA1,TRIM24,RPL7A,SMAD3,SMAD7,TGFB2,RDH5,PML</i> |
| Pregnenolone Biosynthesis | 1.63E-02 | <i>CYP11A1,CYP2E1</i> |
| Tec Kinase Signaling | 1.68E-02 | <i>TNFRSF21,FYN,FOS,JAK1,RND3,RHOB,GNA11,STAT2,GNA14,RHOF</i> |
| Molecular Mechanisms of Cancer | 1.96E-02 | <i>FYN,JAK1,SMAD3,GNA11,SMAD7,GNA14,CDKN2B,FOS,RHOB,RND3,RASGRP1,TGFB2,HIPK2,RHOF,CHEK2,BCL2L11,BMP1,CASP10</i> |
| Glucocorticoid Receptor Signaling | 2.04E-02 | <i>POLR2J2/POLR2J3,SRA1,JAK1,POU2F2,SMAD3,HSPA1A/HSPA1B,HSPA6,POLR2J,MED14,FOS,CCL2,TGFB2,CEBPA,AGT</i> |
| p53 Signaling | 2.52E-02 | <i>TP53AIP1,HDAC9,SNAI2,GADD45A,PML,HIPK2,CHEK2</i> |
| Regulation of eIF4 and p70S6K Signaling | 2.52E-02 | <i>RPS3A,PPM1L,RPS19,RPS10,RPS15A,RPS25,RPS5,EIF4E,RPS12</i> |

Table 4.4. The 10 most enriched biological functions associated with transcripts identified as differentially regulated in bout 2 vs. bout 1 (P (exercise × bout) < 0.05).

| Biological Functions | P Value | Genes |
|--|----------|---|
| Protein degradation and synthesis | 1.03E-05 | <i>APOA1, CHEK2, CREBL2, DPM2, HSPA1A/HSPA1B, JAK1, LAMP2, NACA, PML, RNF149, SMAD3, SMAD7, TRIM24, USP4</i> |
| Gastrointestinal and inflammatory disease | 2.40E-04 | <i>OPRD1, OPRK1, SMAD7</i> |
| Cellular assembly and organization | 2.40E-04 | <i>ABCA1, APOA1, HTT</i> |
| Cell death and survival | 2.42E-04 | <i>BCL2L11, CHEK2, DFFB, FOS, FYN, HIPK2, HTT, KIAA0101, KLF13, MECOM, NQO1, PML, SLC29A2, SMAD3, SMAD7, SMPD1, SNAI2, TRAF3</i> |
| Tissue development | 2.62E-04 | <i>AGT, ANPEP, BCR, BTC, BTG1, CCL2, CCNF, CDKN2B, CEACAM1, CEBPA, CSNK1A1, CYR61, FOS, FOSL1, FTH1, FYN, GADD45A, GEM, HAS2, HBEGF, HIPK2, HMMR, HSPA1A/HSPA1B, IGFBP5, KSR1, MECOM, MET, PAWR, PML, RET, RHOB, SMAD3, SMAD7, SMPD1, SNAI2, SOX8, TGFB2, TRAF3, TRIM24, VTN</i> |
| Gene expression | 3.25E-04 | <i>CDKN2B, CRHR2, EIF4E, FOS, HBS1L, HTT, IGFBP5, JAK1, NACA, POU2F2, RBM4, RPL13A, RPL30, RPL37, RPL39, RPL41, RPS3A, RPS5, RSF1</i> |
| Cell death and survival | 3.62E-04 | <i>BCL2L11, CHEK2, DFFB, FOS, FYN, HIPK2, HTT, KIAA0101, KLF13, MECOM, NQO1, PML, SLC29A2, SMAD3, SMAD7, SMPD1, SNAI2</i> |
| Cellular development, growth and proliferation | 4.40E-04 | <i>ADCYAP1, ANPEP, APOA1, BCR, BTG1, CCL2, CDKN2B, CEACAM1, CEBPA, CLEC4G, CORT, CSNK1A1, CTSZ, DOCK2, DUSP10, FOS, FYN, GADD45A, GRM5, HIPK2, HSPA1A/HSPA1B, IFNK, IGHG1, IL1R1, IL27RA, JAK1, LPIN1, MECOM, MET, MYBL1, PAWR, PCYT1A, PMEL, PML, POU2F2, RASGRP1, RPS19, S100B, SMAD3, SMAD7, SNAI2, TACC3, TGFB2, TLX1, TNFRSF11A, TNFRSF21, TRAF3</i> |
| Tissue development | 4.62E-04 | <i>NACA, ROR2, SMAD3, TGFB2, VTN</i> |
| Inflammatory response and tissue development | 4.70E-04 | <i>IGHG1, SMAD3, SMAD7</i> |

Table 4.5. Top 10 biological functions altered in transcript clusters with different filtering criteria.

| Biological Functions | P Value | Genes |
|---|----------|--|
| Cluster A (54 transcripts, up-regulated in bout 1, P (CON 1 vs. CON 2) < 0.05) | | |
| Cellular development, hematological system development and function, hematopoiesis | 1.48E-03 | <i>FOS,TACC3,TGFB2</i> |
| Gene expression | 1.59E-03 | <i>EIF4E,FOS,RPL41,RPS5</i> |
| Cell cycle, DNA replication, recombination, and repair | 1.92E-03 | <i>EIF4E</i> |
| Cellular development, growth and proliferation, skeletal and muscular system development and function, tissue development | 1.92E-03 | <i>TGFB2</i> |
| Cell cycle | 1.92E-03 | <i>FOS</i> |
| Gene expression | 1.92E-03 | <i>FOS</i> |
| Cell morphology, hematological system development and function, inflammatory response | 1.92E-03 | <i>RHOB</i> |
| Cellular assembly and organization, cellular function and maintenance | 1.92E-03 | <i>RHOB</i> |
| Cellular movement | 1.92E-03 | <i>EIF4E</i> |
| Cell-to-cell signaling and interaction | 1.92E-03 | <i>RDH5</i> |
| Cluster B (231 transcripts, up-regulated in bout 1, P (CON 1 vs. CON 2) > 0.05) | | |
| Gastrointestinal disease and inflammatory disease | 7.26E-06 | <i>OPRD1,OPRK1,SMAD7</i> |
| Tissue development | 1.90E-05 | <i>BCR,BTG1,CCL2,CEACAM1,CSNK1A1,CYR61,FOSL1,FTH1,GADD45A,HBEGF,HIPK2,HSPA1A/HSPA1B,MET,PAWR,SMAD3,SMAD7,SMPD1,SNAI2,SOX8,TRAF3</i> |
| Organismal injury and abnormalities | 6.91E-05 | <i>APOA1,BCR,CCL2,FUT7,IL1R1,SLC4A1,SMAD3</i> |
| Lipid metabolism, molecular transport, small molecule biochemistry | 8.20E-05 | <i>APOA1,SMPD1</i> |
| Protein degradation and synthesis | 1.07E-04 | <i>APOA1,CHEK2,DPM2,HSPA1A/HSPA1B,RNF149,SMAD3,SMAD7</i> |
| Cell death and survival | 1.20E-04 | <i>ANKRD1,AP2A2,APOA1,BCL2L11,BCR,BMP1,BTG1,CCL2,CEACAM1,CHEK2,CSNK1A1,CSRNP1,CYP11A1,CYR61,DRAXIN,DUSP10,EIF4E,FOSL1,FTH1,GADD45A,GRM5,HBEGF,HDAC9,HIPK2,HSPA1A/HSPA1B,HTT,IL1R1,KIAA0101,KLF13,MET,MGAT3,PAWR,PITX3,RND3,ROR2,RPL37,RPS3A,RRAD,RYBP,SMAD3,SMAD7,SMPD1,SNAI2,SOX8,STAT2,TFPI,TNFRSF21,TRAF3,TRIM10,UBR4</i> |
| Cell morphology | 1.52E-04 | <i>BCR,CCL2,HTT</i> |
| Cell death and survival, tumor morphology | 2.01E-04 | <i>BCL2L11,CEACAM1,CYR61,GADD45A,HBEGF,HIPK2,HSPA1A/HSPA1B,MET,PAWR,RND3,RPS3A,SNAI2,TNFRSF21</i> |
| Cell morphology, nervous system development and function, tissue morphology | 2.45E-04 | <i>HTT,MPZ</i> |
| Dermatological diseases and conditions, inflammatory disease, Inflammatory | 2.45E-04 | <i>OPRD1,OPRK1</i> |

Continued on next page

Table 4.5.—Continued

| Biological Functions | P Value | Genes |
|--|----------|--|
| Cluster C (35 transcripts, down-regulated in bout 1, P (CON 1 vs. CON 2) < 0.05) | | |
| Nucleic acid metabolism, small molecule biochemistry | 1.26E-03 | <i>AK5</i> |
| Hematological disease | 2.52E-03 | <i>MECOM</i> |
| Tissue development | 2.62E-03 | <i>CCNF,MECOM,PLXNA4</i> |
| Cell-to-cell signaling and interaction, immune cell trafficking, inflammatory | 3.77E-03 | <i>MR1</i> |
| Connective tissue disorders, organismal injury and abnormalities, Skeletal and muscular disorders | 3.77E-03 | <i>MCPH1</i> |
| Organ morphology, skeletal and muscular system development and function | 3.77E-03 | <i>MCPH1</i> |
| Organ development, organismal development, tissue development | 4.19E-03 | <i>CCNF,MCPH1,MECOM,PLXNA4</i> |
| Cellular development, hematological system development and function | 5.02E-03 | <i>MR1</i> |
| Nervous system development and | 5.02E-03 | <i>PLXNA4</i> |
| Hematological system development and function, hematopoiesis, tissue morphology | 5.02E-03 | <i>MECOM</i> |
| Cluster D (476 transcripts, down-regulated in bout 1, P (CON 1 vs. CON 2) > 0.05) | | |
| Carbohydrate metabolism, molecular transport | 1.25E-04 | <i>ADCYAP1,CEBPA,H6PD,NQO1,PCYT1A,SLC2A4</i> |
| Carbohydrate metabolism, lipid metabolism, molecular transport, small molecule biochemistry | 2.26E-04 | <i>ABCA1,PCYT1A</i> |
| Cellular development, hematological system development and function, hematopoiesis, tissue development | 6.70E-04 | <i>CEBPA,TNFRSF11A</i> |
| Lipid metabolism, small molecule | 1.33E-03 | <i>CYP2E1,CYP4B1</i> |
| Carbohydrate metabolism, molecular transport, small molecule biochemistry | 1.33E-03 | <i>AGT,NMUR1</i> |
| Molecular transport, small molecule biochemistry | 1.33E-03 | <i>POU2F2,SLC47A1</i> |
| Nucleic acid metabolism, small molecule biochemistry | 1.63E-03 | <i>ADH5,H6PD,NQO1</i> |
| Cell death and survival | 2.19E-03 | <i>CNTFR,IL27RA</i> |
| Immunological disease, organismal injury and abnormalities | 2.33E-03 | <i>IL27RA,KIF11,RET</i> |
| Amino acid metabolism, molecular transport, small molecule biochemistry | 3.15E-03 | <i>ADCYAP1,AGT,S100B,SLC6A1,SLC7A13</i> |

IPA network analysis revealed two top networks display a score of 47. Below are the top diseases and functions associated with each of the two top networks: network 1: cell morphology, connective tissue development and function, connective tissue disorders; network 2: gene expression, protein Synthesis, cellular development. Of particular interest is the inclusion of NF- κ B in the network 1. In Figure 4.1, I simplified this network to more clearly illustrate the literature-based relationships of a subset of differentially regulated genes associated with NF- κ B family of transcription factors. I then used IPA to build a network based on known associations between NF- κ B and the differentially expressed genes in our dataset. Table 4.6 presents a list of transcripts that were differentially altered in bout 1 after eccentric exercise, differentially regulated when comparing bout 2 to bout 1, and identified by IPA as having a known association with the NF- κ B transcription factor complex. Taken together, the IPA network analysis implied that the alteration of NF- κ B signaling pathway may be an important molecular event in the CRBE.

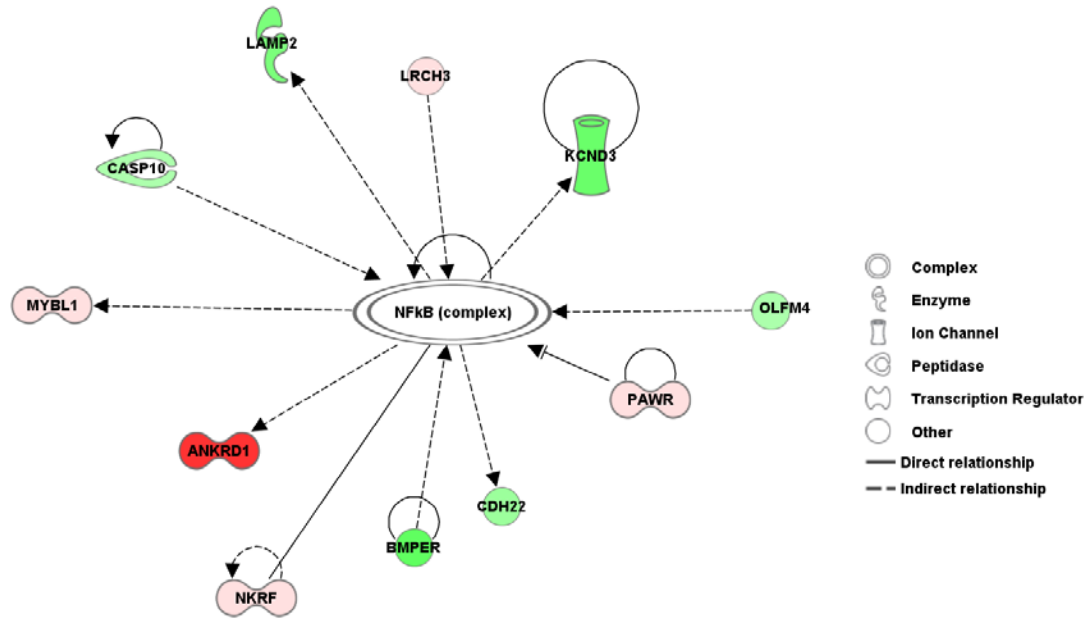


Figure 4.1. The simplified top IPA network of literature-based interactions among genes that were differentially regulated after eccentric exercise in the contralateral leg in bout 2 compared to bout 1 suggests the involvement of NF- κ B in the CRBE. Each colored transcript was altered in bout 1 after eccentric exercise. Transcripts of the NF- κ B complex were not differentially regulated in both bouts. Red signifies an up-regulation of the transcript and green signifies down-regulation after eccentric exercise in bout 1. The intensity of the color corresponds to the magnitude of the fold change. Fold change is indicated below each node. An arrowhead indicates that the protein acted upon by the other connecting node. See Table 4.6 for definition of protein abbreviation. All transcripts were significant at $P(\text{exercise} \times \text{bout}) < 0.05$.

Table 4.6. NF- κ B related genes that were differentially expressed between bout 2 vs. bout 1 (P (exercise \times bout) < 0.05)

| Gene Symbol | Entrez Gene Name | Type | Bout 1 (ECC vs. CON) | | Bout 2 (ECC vs. CON) | | P (exercise \times time) | Relationship to NF- κ B |
|----------------|---|-------------------------|-------------------------|---------|-------------------------|---------|----------------------------------|---|
| | | | Fold Change | P | Fold Change | P | | |
| <i>ANKRD1</i> | Ankyrin repeat domain 1 (cardiac muscle) | transcription regulator | 103.4 | < 0.001 | 41.0 | < 0.001 | 0.012 | NF- κ B is involved in expression of ANKRD1 mRNA |
| <i>CYR61</i> | Cysteine-rich, angiogenic inducer, 61 | other | 28.1 | < 0.001 | 11.9 | < 0.001 | 0.007 | NF- κ B is involved in expression of CYR61 mRNA |
| <i>FOS</i> | FBJ murine osteosarcoma viral oncogene homolog | transcription regulator | 15.1 | < 0.001 | 5.3 | < 0.001 | 0.026 | NF- κ B increases transcription of c-fos (FOS) gene |
| <i>CCL2</i> | Chemokine (C-C motif) ligand 2 | cytokine | 13.1 | < 0.001 | 7.6 | < 0.001 | 0.041 | NF- κ B involved in expression of CCL2 mRNA |
| <i>HBEGF</i> | Heparin-binding EGF-like growth factor | growth factor | 9.9 | < 0.001 | 6.5 | < 0.001 | 0.036 | HBEGF inhibits cytokine-induced NF- κ B activation |
| <i>GADD45A</i> | Growth arrest and DNA-damage-inducible, alpha | other | 7.8 | < 0.001 | 4.1 | < 0.001 | 0.016 | In hibition of NF- κ B increases expression of GADD45A |
| <i>IL1R1</i> | Interleukin 1 receptor, type I | transmembrane receptor | 7.7 | < 0.001 | 4.0 | < 0.001 | 0.003 | IL1R1 protein is involved in activation of NF- κ B |
| <i>TGFB2</i> | Transforming growth factor, beta 2 | growth factor | 7.0 | < 0.001 | 5.0 | < 0.001 | 0.047 | TGFB2 increases activation of NF- κ B |
| <i>TRAFD1</i> | TRAF-type zinc finger domain containing 1 | other | 4.0 | < 0.001 | 2.6 | < 0.001 | 0.031 | TRAFD1 protein decreases activation of NF- κ B |
| <i>HDAC9</i> | Histone deacetylase 9 | transcription regulator | 3.3 | < 0.001 | 1.8 | < 0.001 | 0.004 | NF- κ B is involved in expression of HDAC9 mRNA |
| <i>STAT2</i> | Signal transducer and activator of transcription 2 | transcription regulator | 3.0 | < 0.001 | 1.8 | 0.001 | 0.029 | NF- κ B is involved in phosphorylation of STAT2 protein |
| <i>MET</i> | MET proto-oncogene, receptor tyrosine kinase | kinase | 2.7 | < 0.001 | 1.8 | < 0.001 | 0.023 | MET activates NF- κ B |
| <i>TFPI</i> | Tissue factor pathway inhibitor | other | 2.7 | < 0.001 | 2.0 | < 0.001 | 0.037 | TFPI protein decreases activation of NF- κ B |
| <i>LRCH3</i> | Leucine-rich repeats and calponin homology (CH) domain containing 3 | other | 2.6 | < 0.001 | 1.7 | < 0.001 | 0.009 | Interference of LRCH3 mRNA via siRNA decreases activation of NF- κ B |
| <i>SMAD3</i> | SMAD family member 3 | transcription regulator | 2.2 | < 0.001 | 1.7 | < 0.001 | 0.020 | Inhibition of NF- κ B decreases expression of SMAD3 protein |
| <i>CSNK1A1</i> | Casein kinase 1, alpha 1 | kinase | 2.1 | < 0.001 | 1.6 | < 0.001 | 0.033 | CSNK1A1 increases activation of NF- κ B |
| <i>SMAD7</i> | SMAD family member 7 | transcription regulator | 1.8 | < 0.001 | 1.1 | 0.501 | 0.006 | NF- κ B is involved in expression of SMAD7 mRNA |
| <i>TRAF3</i> | TNF receptor-associated factor 3 | other | 1.4 | 0.001 | 1.0 | 0.905 | 0.034 | NF- κ B increases expresion of TRAF3 mRNA |
| <i>SMPD1</i> | Sphingomyelin phosphodiesterase 1 | enzyme | 1.3 | 0.026 | -1.1 | 0.514 | 0.050 | Interference of SMPD1 via siRNA decreases activation of NF- κ B |
| <i>AGT</i> | Angiotensinogen | growth factor | -1.3 | < 0.001 | 1.0 | 0.750 | 0.015 | NF- κ B increases expression of AGT |
| <i>RET</i> | Ret proto-oncogene | kinase | -1.4 | 0.015 | 1.1 | 0.627 | 0.049 | Mutant RET protein is involved in activation of NF- κ B |

(Continued on the next page)

Table 4.6.—Continued

| Gene Symbol | Entrez Gene Name | Type | Bout 1 (ECC vs. CON) | | Bout 2 (ECC vs. CON) | | P (exercise × time) | Relationship to NF-κB |
|----------------|---|-------------|-------------------------|---------|-------------------------|-------|---------------------------|---|
| | | | Fold Change | P | Fold Change | P | | |
| <i>ATG16L1</i> | Autophagy related 16-like 1 | other | -1.5 | 0.015 | 1.3 | 0.178 | 0.009 | ATG16L1 protein increases activation of NF-κB |
| <i>SLC2A4</i> | Solute carrier family 2 (facilitated glucose transporter), member 4 | transporter | -1.5 | < 0.001 | 1.0 | 0.983 | 0.014 | NF-κB is involved in expression of SLC2A4 protein |
| <i>CYP2E1</i> | Cytochrome P450, family 2, subfamily E, polypeptide 1 | enzyme | -1.6 | < 0.001 | 1.1 | 0.611 | 0.006 | CYP2E1 protein increases activation of NF-κB |
| <i>LAMP2</i> | Lysosomal-associated membrane protein 2 | enzyme | -1.7 | < 0.001 | -1.2 | 0.109 | 0.036 | NF-κB activation mediates expression of LAMP2 protein |
| <i>KCND3</i> | Potassium voltage-gated channel, Shal-related subfamily, member 3 | ion channel | -1.9 | < 0.001 | -1.2 | 0.122 | 0.019 | Inhibition of NF-κB decreases expression of KCND3 |
| <i>IFNK</i> | Interferon, kappa | cytokine | -2.0 | < 0.001 | -1.3 | 0.052 | 0.022 | NF-κB increases induction of IFNK protein |
| <i>BMPER</i> | BMP binding endothelial regulator | other | -2.0 | < 0.001 | -1.2 | 0.347 | 0.027 | BMPER protein increases activation of NF-κB |

qRT-PCR Confirmation of the Expression Changes in the Selected Genes: To

validate microarray data, qRT-PCR was conducted to measure five selected genes (*CCL2*, *ANKRD1*, *IL1R1*, *CYR61*, and *TGFβ2*). These genes were chosen for qRT-PCR confirmation for the following reasons: 1) microarray data showed that they were all differentially expressed in bout 2 compared to bout 1 with a P (exercise × bout) < 0.05; 2) they had a relatively large change in the magnitude of expression after eccentric exercise in bout 1 (see Table 4.6); 3) IPA showed that they all have a known relationship with NF-κB; and 4) they are all related to an inflammatory response. Figure 4.2 displays the results of qRT-PCR. All selected transcripts were up-regulated in the ECC vs. CON leg at bout 1: *CCL2* (12.9-fold), *ANKRD1* (115.7-fold), *IL1R1* (6.9-fold), *CYR61* (26.4-fold), *TGFβ2* (2.7-fold); P < 0.0001 and at bout 2: *CCL2* (9.9-fold), *ANKRD1* (49.1-fold), *IL1R1* (3.5-fold), *CYR61* (13.0-fold), and *TGFβ2* (2.7-fold); P < 0.0001. *ANKRD1* (58%

attenuated increase from bout1 to bout2), *IL1R1* (49% attenuated increase from B1 to B2), and *CYR61* (51% attenuated increase from B1 to B2) demonstrated blunted gene expression responses to eccentric exercise between bouts.

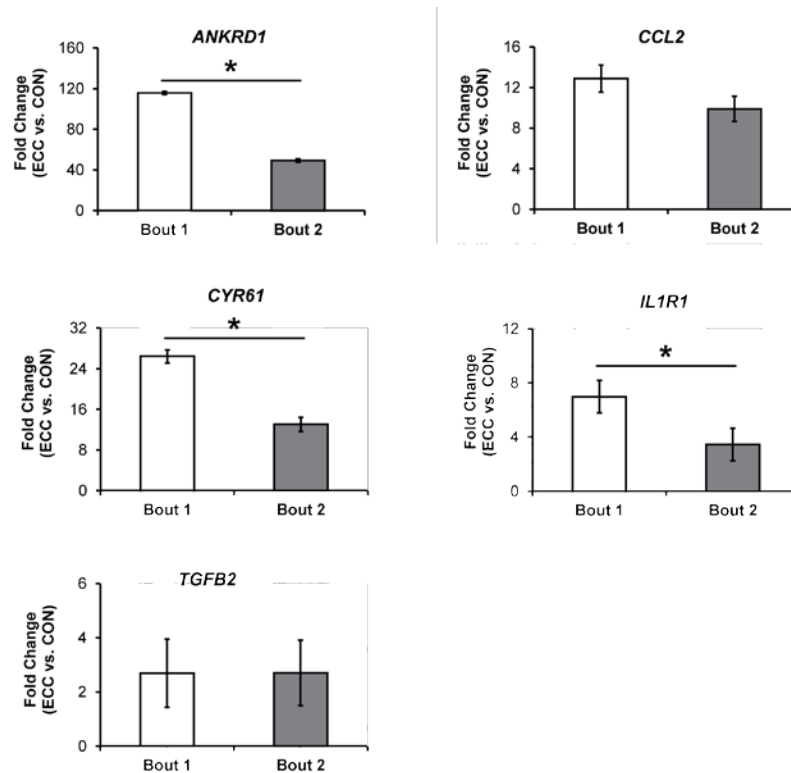


Figure 4.2. qRT-PCR analysis of the five selected genes identified from microarray analysis (N= 17 subjects). Data are presented in fold change (ECC vs. CON leg) as mean \pm SEM. * Gene expression response to eccentric exercise differed significantly between bout 2 versus bout 1 ($P < 0.05$).

Changes of I κ B α Phosphorylation After Two Eccentric Exercise Bouts:

Phosphorylation of I κ B α and subsequent degradation of I κ B α via the ubiquitin/proteasome pathway are critical steps for NF- κ B activation. Because the phosphorylation and degradation processes occur in the cytoplasm, I used Western blot to

assess the amount of I κ B α and p-I κ B α in the cytoplasmic extracts from the muscle biopsy samples collected at 3 h post-exercise in both exercise bouts for 21 subjects. Figure 4.3 depicts the representative Western blot images and respective densitometry bar graphs of I κ B α and p-I κ B α . The statistical analysis showed that there was no significant difference ($P > 0.05$) between the exercised and control leg in either bout 1 or bout 2 for I κ B α . Similarly, p-I κ B α was not significantly ($P > 0.05$) altered after exercise in either bout 1 or bout 2.

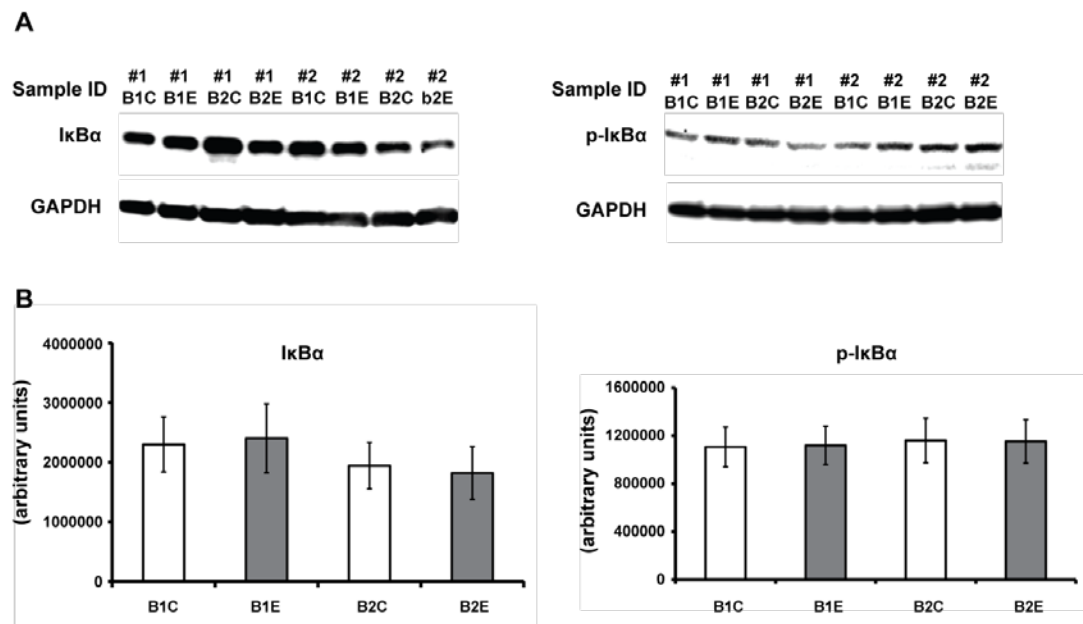


Figure 4.3. Western blot analysis of I κ B α and phosphorylated I κ B α (p-I κ B α) in the cytoplasmic extracts of *vastus lateralis* muscle at 3 h post-exercise after two contralateral bouts of leg eccentric exercise. A) Representative I κ B α and p-I κ B α Western blot images of cytoplasmic extracts of two subjects (#1 and #2) from control (C) or exercised (E) leg after bout 1 (B1) or bout 2 (B2) exercise bout. B) Densitometry of bands in arbitrary units without normalization to the loading control (N = 21). Data are presented as mean \pm SEM.

Discussion

It has been documented that reprogramming of a gene expression profile is important for skeletal muscle adaptation in response to exercise stimuli (38, 140). Microarray is an excellent molecular tool that uses an unbiased approach to identify genes and signaling pathways that contribute to physiological processes. To date, no studies have used microarray analysis to interrogate the genome for differential gene expression changes during the CRBE. In the current study, Agilent whole-genome microarrays were utilized to examine the early changes (3 h post-exercise) in gene expression in the contralateral leg after a second exercise bout compared to the first exercise bout (five weeks between bouts). Therefore, for the first time, our data generated the transcriptome signature of the CRBE and identified 861 transcripts that were significantly altered as a result of the CRBE. The IPA function and network analysis demonstrated that inflammation and NF- κ B are a key biological function and signaling pathway affected by the CRBE. More specifically, we observed significant changes (bout 2 vs. bout 1) in the expression of 27 NF- κ B-related genes that encode cytokines, transcription regulators, growth factors, and transmembrane receptors. Based on the microarray data analysis, I selected five NF- κ B-associated genes (*ANKRD1*, *CRY61*, *IL1R1*, *CCL2*, and *TGFB2*) and used qRT-PCR in an effort to confirm the microarray results. qRT-PCR confirmed that the upregulation of three of these genes (*ANKRD1*, *CRY61*, and *IL1R1*) in response to eccentric exercise was attenuated in bout 2 compared to bout 1.

The *ANKRD1* gene encodes the protein of ankyrin repeat domain 1 (ANKRD1, also known as cardiac ankyrin repeat protein (CARP)) (25). ANKRD1 belongs to the

family of muscle ankyrin repeat proteins (MARPs), along with ankyrin repeat domain protein 2 (ANKRD2/Arpp) and diabetes-related ankyrin repeat protein (DARP) (106). It has been proposed that ANKRD1 may act as a messenger that links mechanosensory processes in the cytoplasm to gene expression regulation in the nucleus (72). ANKRD1 is also involved in inflammation as it is an inducible protein in response to the pro-inflammatory cytokine, tumor necrosis factor α (TNF- α), in human endothelial cells (25). The ANKRD1 gene (*ANKRD1*) has been shown to be a target of NF- κ B (108). Previous studies (23, 80, 95) have demonstrated that *ANKRD1* expression was up-regulated in human skeletal muscle after eccentric exercise. Collectively, these data (23, 57, 80, 95, 108) suggest that NF- κ B is the signaling pathway that up-regulates ANKRD1 gene expression after eccentric exercise.

Cysteine-rich protein 61 (CYR61) is a component of the extracellular matrix and several studies have provided data supporting the hypothesis that CYR61 is a pro-inflammatory factor (5, 75, 76, 175). In murine macrophages, CYR61 has been shown to induce a pro-inflammatory genetic program that includes the increase in pro-inflammatory cytokines (TNF- α , IL-1 α , IL-1 β , IL-6, etc.) and chemokines (e.g. MIP-1 α ; MCP-3) gene expression, as well as down-regulating genes encoding anti-inflammatory cytokines (e.g. TGF- β 1) (5). Zhu et al. (175) has reported that CYR61 contributes to the pathogenesis of rheumatoid arthritis as a pro-inflammatory factor by promoting IL-8-mediated neutrophil infiltration, which plays an important role in the development and severity of rheumatoid arthritis. In addition, Lai et al. (75) observed that CYR61 is pro-inflammatory in obstructive kidney fibrosis via inducing monocyte chemo-attractant protein 1 (MCP-1) and inhibiting CYR61 with a neutralizing antibody attenuated renal

inflammation following ischemic kidney injury (76). Furthermore, several studies indicated that CYR61 activates NF- κ B expression/activity in murine macrophages (5), ovarian cancer cells (79), and retinal vascular endothelial cells (174). CYR61 was shown to be induced 10.1-fold between 4 and 8 h post-high force eccentric exercise in humans (23).

The *IL1R1* gene encodes the interleukin 1 receptor, type 1 (IL-1R1) which is the only receptor for IL-1, binding both IL-1 α and IL-1 β . IL-1 is a prototypical pro-inflammatory cytokine and is associated with a variety of acute and chronic inflammatory conditions (31). IL-1R1 plays a critical role in leukocyte recruitment during the inflammatory response as evidenced by the global attenuation of leukocyte infiltration in IL-1R1 null mice (149). Malm et al. (96) observed an increase in IL-1 β in human skeletal muscle after eccentric cycling exercise. We (57), and others have reported the increase in the expression of the gene *IL1R1* after eccentric exercise in humans (23, 95). The up-regulation of IL-1 and IL-1R1 clearly implicates the involvement of the pro-inflammatory IL-1 signaling pathway in the inflammatory response after eccentric exercise. Blocking of the IL-1R1 protein using a neutralizing antibody decreases activation of NF- κ B in response to ethanol in cultured rat astrocytes, indicating IL-1R1 as an upstream activator of NF- κ B (12).

Given the aforementioned pro-inflammatory properties of ANKRD1, CRY61, IL1R1, and their positive relationship (either being an upstream activator or a downstream target gene of NF- κ B) with NF- κ B, the induction of genes encoding all these three proteins in the present study at 3 h post-eccentric exercise in both exercise bouts implicates an acute inflammatory response involving NF- κ B after eccentric exercise. The

diminished induction of these genes in bout 2 vs. bout 1 in the current study suggests an attenuated acute inflammatory response mediated by NF- κ B. Since an excessive acute inflammatory response plays a key role in EIMD (24, 74, 160), it is reasonable to propose that the more modest NF- κ B mediated inflammatory response in the contralateral leg in bout 2 may be a mechanism responsible for the CRBE.

In contrast to our hypothesis, we did not identify any significant changes in total I κ B α or phosphorylated I κ B α either after either bout of eccentric exercise or between two exercise bouts. One possible reason for this may be the 3 h time point we chose for these studies. It has been reported that phosphorylation of IKK α/β returns to basal levels by 3 h post-submaximal treadmill running exercise in rats (47). Similarly, I κ B α and p-I κ B α returned to pre-exercise levels 4 h after exhaustive treadmill running in rats (63). Therefore, we may have missed the optimal time point to observe changes in I κ B α and p-I κ B α . Future studies are warranted to examine the time-course changes in the upstream regulators of NF- κ B (phosphorylation of IKK α/β and I κ B α).

Based on our data in the present study, and previously reported neural adaptation in the CRBE, we proposed a model to explain the CRBE (Figure 4.4). According to our model, there is an optimization of motor unit recruitment during bout 2 exercise based on previous results (21, 155), which leads to more modest mechanical damage to the muscle. Since pro-inflammatory cytokine secretion is likely proportional to the extent of muscle damage, then presumably fewer leukocytes will be recruited to infiltrate into the damaged muscle areas. A decrease in CYR61, IL-1R1, and cytokines results in lower IKKs phosphorylation (p-IKKs), which means reduced levels of degradation of I κ B. Therefore, less NF- κ B is activated to translocate to the nucleus to drive the expression of target

genes encoding for pro-inflammatory mediators (e.g., ANKRD1, IL-1, CCL2, and TGFβ2). The end result is a reduced inflammatory response producing less secondary damage and a faster recovery.

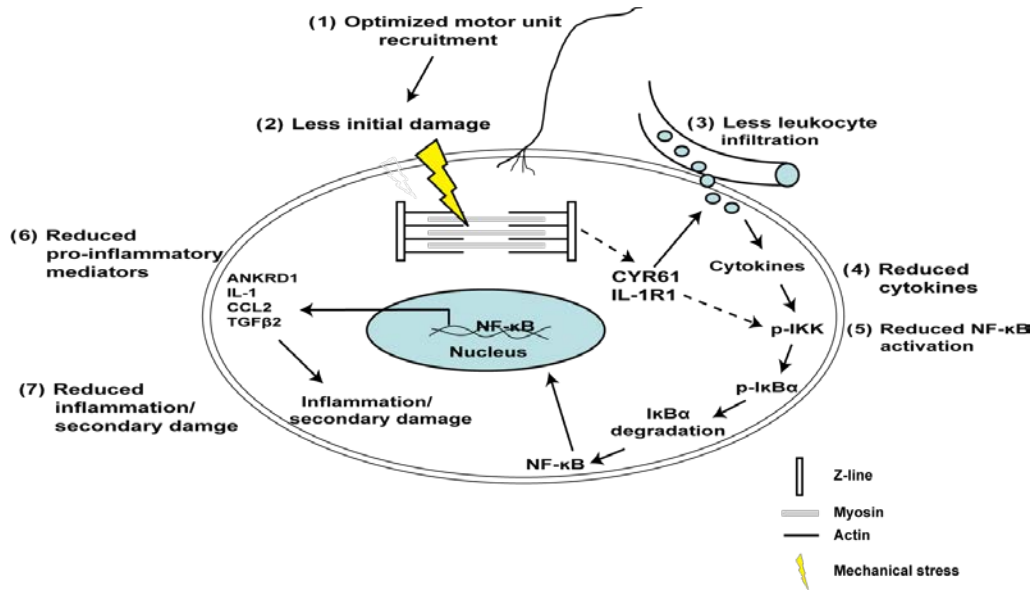


Figure 4.4. Proposed model for the regulatory mechanism(s) responsible for the contralateral repeated bout effect (CRBE). Solid arrows indicate known processes and dashed arrows demonstrate unknown signaling cascade. See detailed explanation in the texts.

There are two major limitations to this study. First, there are potential confounding effects from the supplements with antioxidant and anti-inflammatory properties. Although no significant differences were found between the placebo and supplement groups for all functional parameter measurements (CK, soreness, and muscle strength loss), the supplements may have small non-significant effects on the inflammation-related signaling molecules/pathways such as NF-κB pathway. Second, we

only examined the transcriptomic changes at 3 h post-exercise to avoid performing too many muscle biopsies on the subjects, which may have prevented us from identifying other important signaling genes/pathways whose changes might be only significant at a later time point post-exercise.

In conclusion, this study provides the first transcriptomic analysis of the CRBE and lays the groundwork for future studies focused on defining the molecular and cellular mechanisms responsible for the CRBE. Furthermore, the findings of the current study expanded our previous report (173) and further strengthens our hypothesis that an attenuated inflammatory response involving NF- κ B is probably a key mechanistic basis for the CRBE. Therefore, our data define the involvement of NF- κ B and its associated key pro-inflammatory mediators (ANKRD1, CYR61, IL1R1) in the CRBE, suggesting that inhibition of NF- κ B mediated inflammatory pathway may be useful for optimizing CRBE while setting up rehabilitation exercise for a unilaterally immobilized limb.

CHAPTER 5

STUDY III

ANKYRIN REPEAT DOMAIN 1 (ANKRD1) IS A POTENTIAL MARKER OF SKELETAL MUSCLE DAMAGE INDUCED BY ECCENTRIC EXERCISE AND OTHER PATHOLOGICAL CONDITIONS

Abstract

Ankyrin repeat domain 1 (ANKRD1) is a mechanosensing protein in skeletal muscle. To determine if ANKRD1 is a potential biomarker for skeletal muscle damage, I examined ANKRD1 expression in several muscle damage paradigms. In one paradigm, young men performed eccentric exercise with one leg and biopsy samples from the *vastus lateralis* were collected from both the control (CON) and eccentric-exercised (ECC) leg at 3 h post-exercise. ANKRD1 mRNA levels were analyzed via microarray and quantitative real-time PCR (qRT-PCR). I found that ANKRD1 mRNA was up-regulated at 3 h post-eccentric exercise (fold change (FC) = 103.4 ± 1.3 (microarray) and 115.7 ± 1.6 (qRT-PCR), $P < 0.001$). In the second paradigm, young men performed eccentric exercise with one leg and biopsies were obtained pre-exercise from the CON leg and 24 h post-exercise from the ECC leg and analyzed for ANKRD1 expression at the protein level. Compared to the CON leg, the ECC leg had a 1.2-fold increase in cytoplasmic ANKRD1 protein ($P = 0.002$) at 24 h post-exercise. Nuclear ANKRD1 protein levels in the same individuals 24 h post-exercise were positively correlated with peak muscle strength loss post-exercise ($r = 0.88$, $P = 0.007$). Finally, using Gene Expression Omnibus (GEO) profile data, I analyzed ANKRD1 mRNA expression from a variety of other muscle damage models, including: Duchenne muscular dystrophy, dystrophin-

deficient mdx mice, freeze-induced muscle injury in mice, α 2-laminin deficient muscular dystrophy, and Infantile-onset Pompe disease. ANKRD1 mRNA was up-regulated in all of these muscles relative to unaffected individuals ($P < 0.01$). The consistent increase in ANKRD1 mRNA across various skeletal muscle damage models, and the positive correlation between ANKRD1 protein and muscle strength loss, suggest that ANKRD1 is a potential muscle damage biomarker.

Keywords: muscle damage, ANKRD1, eccentric exercise, nuclear translocation

Introduction

Skeletal muscle is an extremely plastic tissue that adapts to a variety of stress stimuli, including exercise, denervation, starvation, and hypoxia (39). Each of these different physiological challenges activates signal transduction cascades that ultimately result in muscle remodeling and adaptation (72). Remodeling is an adaptive response to environmental challenges with the goal of maintaining muscle function and involves alterations in biochemical and morphological features of both muscle fibers and associated structures such as motoneurons and capillaries.

Eccentric (muscle lengthening) exercise induces temporary muscle damage which can be repaired within a couple of weeks post-exercise (55, 57, 173) and therefore represents an effective and reliable model to explore the cellular and molecular mechanisms underlying muscle remodeling and repair after mechanical stress from exercise (56). The most frequently used indirect markers for exercise-induced muscle damage (EIMD) are: muscle strength loss, delayed onset muscle soreness, and the release of muscle-specific proteins into the blood (27). Among these measures, the reduction in

muscle force production post-exercise has been considered to be the best indirect marker of EIMD for several reasons. Firstly, teleologically, a muscle injury marker should be related to muscle function (i.e., the ability of a muscle to produce muscle force) and muscle strength measurement is directly proportional to muscle function. Secondly, it is a non-invasive measure, which means that repeated measurements are easy to obtain throughout the recovery process. In this perspective, muscle force measurement has high reliability (intraclass correlation coefficients ≥ 0.85) (1). Lastly, other EIMD markers, including direct evidence of muscle damage (histological abnormalities), correlate poorly with muscle force reduction post-exercise (168).

Several laboratories, including ours, have used microarray analyses as an unbiased method to identify genes involved in muscle responses to eccentric exercise (9, 23, 57, 95). Data from our EIMD microarray study have identified hundreds of differentially expressed genes at 3 h post-eccentric exercise that are associated with key biological processes, including inflammation, cell growth, and protein synthesis (57). Among those differentially expressed genes, *ANKRD1* was identified as the gene that displays the highest fold change (FC = 103.4) after eccentric exercise. With such a rapid and dramatic change in expression following eccentric exercise, I sought to determine if the *ANKRD1* gene is induced in other muscle injury models/disorders in mammals. If it is, then elevations in the expression of *ANKRD1* gene expression might represent as a universal marker of muscle injury, independent of the nature of the insult.

The *ANKRD1* gene encodes the cardiac ankyrin repeat domain 1 (ANKRD1, also known as cardiac ankyrin repeat protein (CARP)) (25). ANKRD1, together with ankyrin repeat domain protein 2 (ANKRD2/Arpp) and diabetes-related ankyrin repeat protein

(DARP), belongs to a family of conserved muscle specific ankyrin repeat proteins (MARPs) (25, 58, 68, 106). ANKRD1 was discovered in 1995 as a cytokine-inducible nuclear protein in human microvascular endothelial cells (25). Zou et al. (176) demonstrated that ANKRD1 is a transcription cofactor that binding to the Y-box transcription factor 1 (YB-1), where it appears to negatively regulate the transcription of myosin light chain 2 ventricular gene (*MLC-2v*), the earliest marker of cardiac chambers regionalization during mammalian cardiogenesis. ANKRD1 is localized both in the nucleus and the sarcomeric I-band, where it binds to the N2A portion of titin, a key element of the mechanosensor complex in striated muscle (72). Besides binding to YB-1 and titin, ANKRD1 has also been shown to interact with other proteins such as myopalladin (8), desmin (106), calsequestrin-2 (CASQ2) (72), and muscle-specific RING finger proteins (MuRF1 and MuRF2) (172). Given its dual subcellular localization (nucleus and cytoplasm), and its interaction with multiple partners such as titin and transcription factor YB-1, it has been proposed that ANKRD1 participates in sensing a mechanical stress stimulus as an element of the mechanosensor complex in the cytoplasm, and then translocates into the nucleus where it modulates gene expression following injury (106).

To date, only a few studies (23, 80, 95) have reported observing changes in ANKRD1 expression in human skeletal muscle following eccentric exercise. Chen et al. (23) reported that ANKRD1 mRNA was increased 7.5-fold at 4-8 h after eccentric exercise in humans. A later study by Mahoney et al. (95) reported that the ANKRD1 gene was up-regulated 9.7-fold at 3 h and 14-fold at 48 h in humans following a bout of eccentric exercise. In addition, levels of the ANKRD1 gene have been reported to

increase in human *vastus lateralis* muscle as early as 30 min post-exercise (20). These elevated values were still evident two days after a bout of fatiguing jumping exercise that involved an eccentric loading component (80). However, none of these studies measured ANKRD1 expression at the protein level, so it is unknown if there are functional changes in this pathway following EIMD.

Therefore, to determine if ANKRD1 expression is altered during muscle damaging exercise, I performed two studies with human subjects to examine the expression of ANKRD1 at the mRNA and protein levels in skeletal muscles following damaging exercise. I also determined the subcellular distribution of ANKRD1 protein both biochemically and histologically. Analyses were performed to determine if ANKRD1 expression and/or subcellular distribution correlated with muscle strength loss following EIMD. I observed a significant increase in cytoplasmic ANKRD1 protein levels at 24 h post-eccentric exercise. Most importantly, for the first time, my data indicated that ANKRD1 nuclear accumulation is positively correlated with muscle strength loss. Lastly, I used publically available microarray databases to determine if ANKRD1 expression is elevated in a range of different models for muscle damage rodents and humans. I observed that ANKRD1 mRNA was upregulated in all assayed muscle damage models. Data from this study will improve our understanding of the roles of ANKRD1 in the muscle stress response and may also suggest that ANKRD1 has utility as a diagnostic marker for skeletal muscle damage.

Methods

Study Design: Skeletal muscle biopsy samples were collected at 3 h (3h-Study) and 24 h (24h-Study) following exercise from two human studies. The design of 3h-Study has been reported previously (173). Briefly, subjects performed eccentric leg exercises unilaterally (knee extensors) and a muscle biopsy from the control (CON) and eccentric-exercised (ECC) *vastus lateralis* muscle was taken 3 h post-exercise. The 24h-Study consisted of seven laboratory visits. Subjects reported to the laboratory on their first day and began by receiving a familiarization session on the Biodex System 4 dynamometer (Biodex Medical Systems, Shirley, NY). On that visit, baseline muscle strength and soreness were assessed and a pre-exercise control biopsy sample was taken from the *vastus lateralis* muscle that was randomized to be the non-exercised leg. Subjects reported back to the lab 24 h later to perform the eccentric exercise protocol. They then returned to the laboratory 24 h after the completion of the exercise protocol and underwent a second muscle biopsy of the *vastus lateralis* muscle on the exercised leg. Muscle strength was also assessed at this visit. Subjects returned to the laboratory every 24 h for the next 3 days to have their muscle strength assessed.

Subjects: Thirty-five men (20.9 ± 0.5 yrs, 178.9 ± 1.2 cm and 80.6 ± 2.9 kg) volunteered to participate in the 3h-Study and seven men (22.6 ± 2.1 yrs, 180.4 ± 4.1 cm and 73.3 ± 16.4 kg) completed the 24h-Study. All subjects signed a written informed consent form approved by the Institutional Review Board (IRB) of the University of Massachusetts Amherst (3h-Study) or Brigham Young University (24h-Study). A homogenous subject population was chosen to avoid possible confounding effects of some factors that may

influence the response of muscle to exercise such as age, gender, and training status. All subjects were non-smokers, relatively young (aged between 18-30 years old), sedentary using the standard activity level of less than six metabolic equivalent tasks (METs) per day, and healthy with no diagnosis of a medical condition, including but not limited to diabetes, hypertension, kidney, cardiovascular or pulmonary disease. Subjects were excluded if they had participated in weight training activity of the lower body within six months prior to the study or if they were unwilling to refrain from taking analgesics during the study period.

Eccentric Exercise Protocol: The eccentric exercise protocol of the 3h-Study has been described previously (57, 173). Briefly, subjects performed 100 maximal eccentric repetitions of knee extensor at a speed of 30°/sec on a Biodex System 4 isokinetic dynamometer (Biodex Medical Systems, Shirley, NY, USA). In the 24h-Study, subjects performed multiple sets of eccentric contractions of knee extensors at a rate of 120°/sec until approximately 40kJ of work was achieved (196.1 ± 37.2 total repetitions). Sets consisted of approximately 2kJ of work with a one-min rest between sets. During each contraction, subjects resisted as the lever pulled their leg from 40° of knee flexion into approximately 115° of knee flexion. In both studies, subjects were verbally encouraged to provide a maximal effort during each exercise repetition.

Knee Extension Strength Measurement: The protocol of isometric muscle strength measurement was composed of three trials of 3-sec isometric contractions with a one-min

rest between trials at the angle of 70° of knee flexion. The average of the highest isometric torque value of the three trials was defined as the isometric peak torque.

Muscle Biopsies: In the 3h-Study, muscle biopsy samples from the 35 subjects were collected at 3 h post-exercise from both the CON and ECC leg. In the 24h-Study, biopsy samples were obtained pre-exercise from the CON leg and at 24 h post-exercise from ECC leg. Muscle biopsies were taken percutaneously from the *vastus lateralis* muscle of the leg under local anesthesia (2% Lidocaine). A small incision was made into the skin and fascia and then the biopsy needle was inserted into the muscle. In the 3h-Study, a small piece of tissue (about 50-200 mg) was withdrawn and snap-frozen in liquid nitrogen. In the 24h-Study, a small core of tissue (~ 100 mg) was withdrawn. Following withdrawal of the tissue, it was separated from any fatty tissue. The muscle sample was then divided into multiple 25-50 mg portions. Samples that were to be used for biochemical studies were immediately frozen in liquid nitrogen until needed. Portions of tissue designated for sectioning and immunohistochemistry were mounted on a cork with tragacanth gum and frozen in isopentane cooled in liquid nitrogen to avoid ice crystal formation.

RNA Preparation and Microarray Analysis: Muscle biopsy samples from the 3h-Study were homogenized in TRIzol (Invitrogen, Carlsbad, CA, USA) to isolate total RNA. cDNA was prepared using the Agilent Whole Human Genome Oligo Microarray kit (Agilent, Santa Clara, CA) according to the manufacturer's instructions and hybridized to arrays that contained probe sets representative of ~41,000 unique human

genes/transcripts. The RNA isolation, probe generation, and microarray hybridization were performed by Gene Logic Inc. (Gaithersburg, MD).

Quantitative Real-Time PCR (qRT-PCR): I used qRT-PCR as an independent method to confirm ANKRD1 gene induction identified from the microarray analysis. Samples from 17 of the 35 subjects from the 3h-Study had sufficient mRNA to perform qRT-PCR analysis. For each assay, 200 ng of total RNA was reverse transcribed into cDNA using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. cDNA was amplified in triplicates using SsoFastEvaGreen Supermix (Bio-Rad Laboratories Inc, Hercules, CA) on a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories Inc, Hercules, CA). The PCR program included 30s at 95°C for enzyme activation, which was followed by 40 cycles of: 1) 3 sec at 95°C for denaturation; and 2) 5 sec at 60°C for annealing/extension. At the end of the final cycle of each reaction, a melting curve analysis was performed to monitor the EvaGreen fluorescence continuously throughout the temperature range from 65°C to 95°C in 0.5°C increments and a 5-sec hold at each degree. Observation of a single melt peak from each sample was used to rule out the presence of primer dimers. Since the expression of beta-2-microglobulin (B2M) does not change following eccentric exercise (94), it was used as an internal control. All forward and reverse primers for *ANKRD1* (forward: 5'- AAG CGA GAA ACA ACG AGA GGC AGA-3'; reverse: 5'- AGA AAC GTA GGC ACA TCC ACA GGT-3') and β 2 microglobulin (*B2M*) (forward: 5'- TGT CTG GGT TTC ATC CAT CCG ACA -3'; reverse: 5'- TCA CAC GGC AGG CAT ACT CAT CTT -3') were designed using NCBI gene sequences with the PrimerQuest program (Integrated

DNA Technologies, Coralville, IA). Differential gene expression was determined by the relative quantification $\Delta\Delta$ Ct method. Relative quantification of amplified mRNA was normalized to B2M to compensate for variations in quantity and real time efficiency.

Western Blot Analysis: To quantify ANKRD1 protein levels within the muscles, and to determine its subcellular localization, I employed the NE-PER Nuclear and Cytoplasmic Extraction Kit (cat# 78835, Thermo Scientific, Rockford, IL) according to the manufacturer's instructions. Equal amounts (12 μ g per lane for cytoplasmic extract and 10 μ g per lane for nuclear extract) of protein from each sample were fractionated in 4-15% gradient sodium dodecyl sulfate (SDS) polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA). Proteins were then transferred at 4°C (75V for 45 minutes and then 100V for 15 minutes) to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA). The membrane was blocked with 5% dry milk (cat# 170-6404, Bio-Rad Laboratories, Hercules, CA) or 5% Bovine Serum Albumin (BSA, cat# sc-2323, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in 1 \times TBST for 1 h at room temperature and then incubated overnight at 4°C with the appropriate primary antibodies (rabbit anti-ANKRD1, cat# NBP1-55639, 1:5000 in 5% dry milk/TBST (mixture of Tris-Buffered Saline and Tween 20), Novus Biologicals, LLC, CO; rabbit anti-GAPDH, cat# ab9485, 1:10000 in 5% BSA/TBST, Abcam, MA; rabbit anti-Lamin B1, cat# ab16048, 1:2000 in 5% dry milk/TBST, Abcam, MA). The membranes were then incubated with the HRP-conjugated secondary antibodies (goat anti-rabbit IgG (H + L)-HRP conjugate, cat# 170-6515, Bio-Rad Laboratories, Hercules, CA, 1:20000 in 5% BSA/TBST for GAPDH blots; goat anti-mouse IgG (H + L)-HRP conjugate, cat#170-6516, Bio-Rad Laboratories,

Hercules, CA, 1:5000 in 5% dry milk/TBST for p Lamin B1 blots) for 1 h at room temperature. The blots were developed using enhanced chemiluminescence (ECL) and the visualized bands were quantified via densitometry using BioRad ChemiDoc™ XRS+ System with Image Lab™ Software. Densitometry calculations of protein bands allowed for a comparison of the changes in the relative amount of ANKRD1 protein product in the muscle samples.

Immunofluorescence Quantification: Muscle biopsy samples were flash frozen in liquid nitrogen and then serially cross-sectioned to an 8 µm thickness on a Microm HM 505E cryostat at -25°C (Richard Allan Scientific, Kalamazoo, MI), mounted on Superfrost slides (Fisher Scientific, Pittsburgh, PA) and air-dried for 20 min. Negative control (no primary antibodies added) sections were included in all analyses. Sections were fixed in 4% paraformaldehyde (cat# 50-259-99, Fisher Scientific, Pittsburgh, PA) for 15 min at room temperature and then incubated in blocking solution (2% BSA, 5% FBS (cat# s11550, Atlanta Biologicals, Norcross, GA), 0.2% sodium azide) for 30 min at room temperature. All sections, except negative controls, were then incubated in rabbit anti-ANKRD1 primary antibody (NBP1-55639, 1:50 dilution; Novus Biologicals, LLC, CO) diluted in blocking solution overnight at 4°C. On the next day, sections were washed three times in 1×PBS for 5 min and then incubated in fluorescence-conjugated secondary antibody (FITC goat-anti-rabbit, 111-095-003, Jackson ImmunoResearch Laboratories Inc. PA, 1:100 dilution in 1×PBS) for 1 h at 37°C. 4', 6-diamidino-2-phenylindole (DAPI) was added into the secondary antibody solution to stain nuclei. After DAPI and secondary antibody incubation, sections were washed three times in 1×PBS for 5 min.

After the last wash, sections were air-dried and mounted using a drop of Vectashield mounting medium (Vector Laboratories, Burlingame, CA).

Images were captured via an inverted phase contrast microscope (Nikon Eclipse, TE2000-S) equipped with a Spot Insight camera and MetaVue imaging software (version 6.1r5) with a $\times 20$ objective and 30-50 myofibers per field were analyzed using the NIH Image J analysis program. The average density of ANKRD1 staining from three to five randomly chosen fields was measured for each fiber. ANKRD1 staining in the nucleus and cytoplasm were analyzed separately. For cytoplasm stain quantification, the mean fluorescence intensity of ANKRD1 staining within the same size /shape area ($15 \mu\text{m}^2$) in the center of each myofiber was measured and analyzed. The average of the mean ANKRD1 staining intensity per area of all myofibers within each field was calculated and used for statistical analysis. For quantification of nuclear staining, the DAPI staining was subtracted from ANKRD1 staining using the image calculator to define the cytoplasmic ANKRD1 staining. The cytoplasmic image was then subtracted from the original ANKRD1 image to create the nuclear ANKRD1 image. The mean ANKRD1 staining intensity per nucleus within each field was measured and analyzed.

Gene Expression Omnibus (GEO) Profile Analysis: The GEO profiles at NIH (<http://www.ncbi.nlm.nih.gov/geoprofiles/>) were queried for available data sets that included the ANKRD1 probe. I chose five studies of skeletal muscle disorders that included samples from: 1) the quadriceps of healthy individuals or Duchenne muscular dystrophy (DMD) patients (44) (data accessible at NCBI GEO database, accession no. GSE1004); 2) gastrocnemius muscle of wild-type or dystrophin-deficient mdx mice (161)

(GEO accession no. GSE466); 3) tibialis anterior muscle of control or 6 h post-freeze-induced muscle injured mice (10-second freezing using a 6-mm diameter steel probe cooled to the temperature of dry ice (-79°C) (169) (GEO accession no. GSE5413); 4) diaphragm muscle of mice with normal or α 2-laminin deficient muscular dystrophy (165) (GEO accession no. GSE 3252); and 5) biceps muscle of control or Infantile-onset Pompe diseased babies (138) (GEO accession no. GSE38680). Unpaired t-tests were performed to analyze the differences in mRNA levels of ANKRD1 between the normal/wild type control (CON) and injured (INJ) muscle for the five data sets.

Statistical Analysis: Partek Genomics Suite (V6.5, Partek, Inc., St. Louis, MO) was used to analyze the microarray data via ANCOVA (age and BMI covariates) to determine if mRNA levels of ANKRD1 were differentially expressed at 3 h post-eccentric exercise. Paired t test and Pearson's correlation analysis were used to assess the relationship between fold change in nuclear ANKRD1 protein and the percentage (%) of peak muscle strength loss (the peak muscle strength loss occurred 48 h post-eccentric exercise in the 24h-Study). Data are expressed as means \pm standard error (SEM) and statistical significance was set at $P < 0.05$.

Results

Induction of ANKRD1 Gene Expression at 3 h Post-Eccentric Exercise: Using microarray analysis, I compared the expression level of ~41000 transcripts between the ECC leg vs. CON leg. The full dataset can be accessed via the NCBI GEO database (accession no. GSE23697). Among the genes that were differentially expressed at 3 h

post-exercise, *ANKRD1* displayed the greatest fold change in expression ($FC = 103.4 \pm 1.3$, $P < 0.001$, Figure 5.1). To validate this observation, I performed qRT-PCR with primers to *ANKRD1* with beta-2-microglobulin (*B2M*) as a control. In good agreement with the array data, I observed that *ANKRD1* gene expression increased more than 100 fold by 3h post-exercise ($FC = 115.7 \pm 1.6$, $P < 0.001$, Figure 5.1).

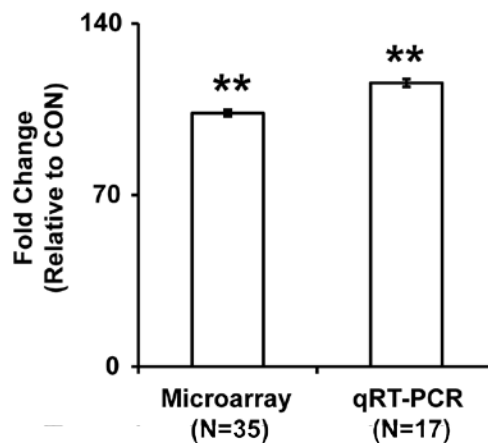


Figure 5.1. Induction of *ANKRD1* mRNA in human *vastus lateralis* at 3 h following eccentric exercise. *ANKRD1* mRNA levels were quantified by microarray and confirmed by qRT-PCR experiments. Data are presented as mean \pm SEM. ** $P < 0.001$ (ECC vs. CON leg).

Nuclear translocation of ANKRD1 at 24 h Post-Eccentric Exercise Was Positively

Associated With Peak Muscle Strength Loss: Since *ANKRD1* may function as a

transcriptional regulator, I wanted to determine both the level and localization of

ANKRD1 in control and eccentrically exercised human skeletal muscle. Consequently,

we isolated proteins from biopsy samples from the 24h-Study and separately analyzed the

nuclear and cytoplasmic fractions using Western blotting. In each case, I presented paired

samples from the same individuals to facilitate comparison of the control and exercised muscle.

I chose GAPDH as the loading control for the cytoplasmic extracts because it has been used as a loading control for Western blot analysis in eccentric exercise models since it does not change in response to eccentric exercise (88). I used Lamin B1, a nuclear envelope protein, as a loading control for the nuclear extracts.

Grossly, I did not observe any change in ANKRD1 protein levels in the cytoplasm of the paired samples, an observation that was confirmed by densitometry (Fig 3B). In contrast to the data with cytoplasmic ANKRD1, for three of the seven individuals there was a dramatic increase in the levels of nuclear ANKRD1 following exercise (Figure 5.2A). In the aggregate, there was a 2.9-fold increase in nuclear ANKRD1 in the exercised samples relative to the controls (FC = 2.9, Figure 5.2B). Nevertheless, the change did not reach statistical significance ($P > 0.05$) for either cytoplasmic or nuclear ANKRD1 abundance (Figure 5.2B), probably due to the high inter-subject variations and relatively small sample size ($N = 7$). (It should be noted that the lamin B1 band intensity was not consistent across all lanes (Figure 5.2B), perhaps due to issues related to the transfer.

Interestingly, it was noticed that the subjects with the greatest change in nuclear ANKRD1 protein were also the subjects that lost the most muscle strength. Therefore, I examined the correlation between the isometric muscle strength loss values (% of CON) (at 2 d post exercise - which was the peak for most subjects) and the fold change in nuclear and cytoplasmic ANKRD1 from Western blots using a Pearson's correlation analysis. There was a significantly positive relationship between muscle strength loss and

nuclear ANKRD1 increase ($r = 0.88$, $P = 0.007$, Figure 5.2C). In contrast, there was no significant correlation was identified between muscle strength loss and cytoplasmic ANKRD1 expression (Figure 5.2C).

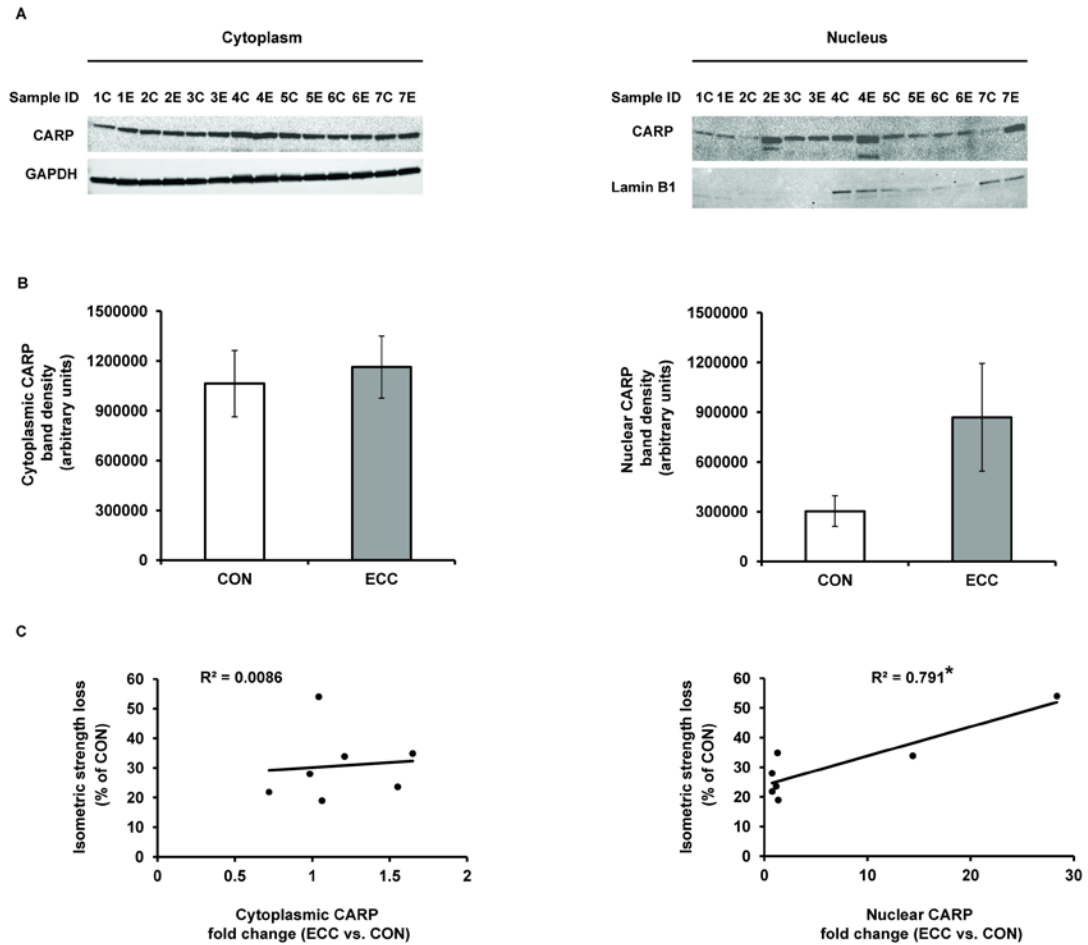


Figure 5.2. Western blot analysis of protein levels of ANKRD1 in the cytoplasmic and nuclear fractions of *vastus lateralis* muscle at 24 h post-eccentric exercise and the correlation between ANKRD1 protein content and muscle strength loss. A) Western blots of cytoplasmic or nuclear samples of seven subjects (#1-7) from control (C) or exercised (E) leg with GAPDH and Lamin B1 as the cytoplasmic and nuclear loading control, respectively. B) Raw densitometry of cytoplasmic or nuclear protein bands in arbitrary units without normalizing over loading controls C) Pearson's correlation of isometric muscle strength loss with cytoplasmic or nuclear ANKRD1 protein content changes (fold change of ECC vs. CON leg). Data are presented as mean \pm SEM. * $P < 0.05$.

Cytoplasmic ANKRD1 Immunoreactivity Increases at 24 h Post-Exercise: To further analyze ANKRD1 protein expression, and to obtain better spatial resolution than can be achieved with biochemical methods, I used immunofluorescence to determine if ANKRD1 protein levels were in the cytoplasm and/or nucleus following exercise. (Only the biopsy samples from the 24 h-Study were used for immunofluorescence analysis because the samples from the 3h-Study had ice crystal freezing artifacts, which could confound anatomical studies). For this analysis, I computationally placed a 15 μm^2 circle randomly near the center of each fiber viewed under the microscope and measured the fluorescence intensity. In total, I analyzed 1365 myofibers (638 control fibers and 727 exercised fibers). ANKRD1 staining was predominantly localized to the cytoplasm and with minimal staining in the nuclear area (Figure 5.3A-I). The immunofluorescence intensity of ANKRD1 in the cytoplasm was significantly ($P = 0.008$) increased in the exercised sample as compared to the contralateral control sample (Figure 5.3J) suggesting that cytoplasmic ANKRD1 protein was induced at 24 h post-eccentric exercise. Unexpectedly, there was no significant difference in the nuclear ANKRD1 staining intensity between ECC and CON samples (data not shown). I did notice some truncated areas that were stained positive for ANKRD1 but negative for DAPI staining (arrowheads in Figure 5.3B, C, H, I). Based on the location and DAPI-negative staining of these areas, it is possible that they are cytoplasmic areas of non-muscle cells such as pericytes or endothelial cells.

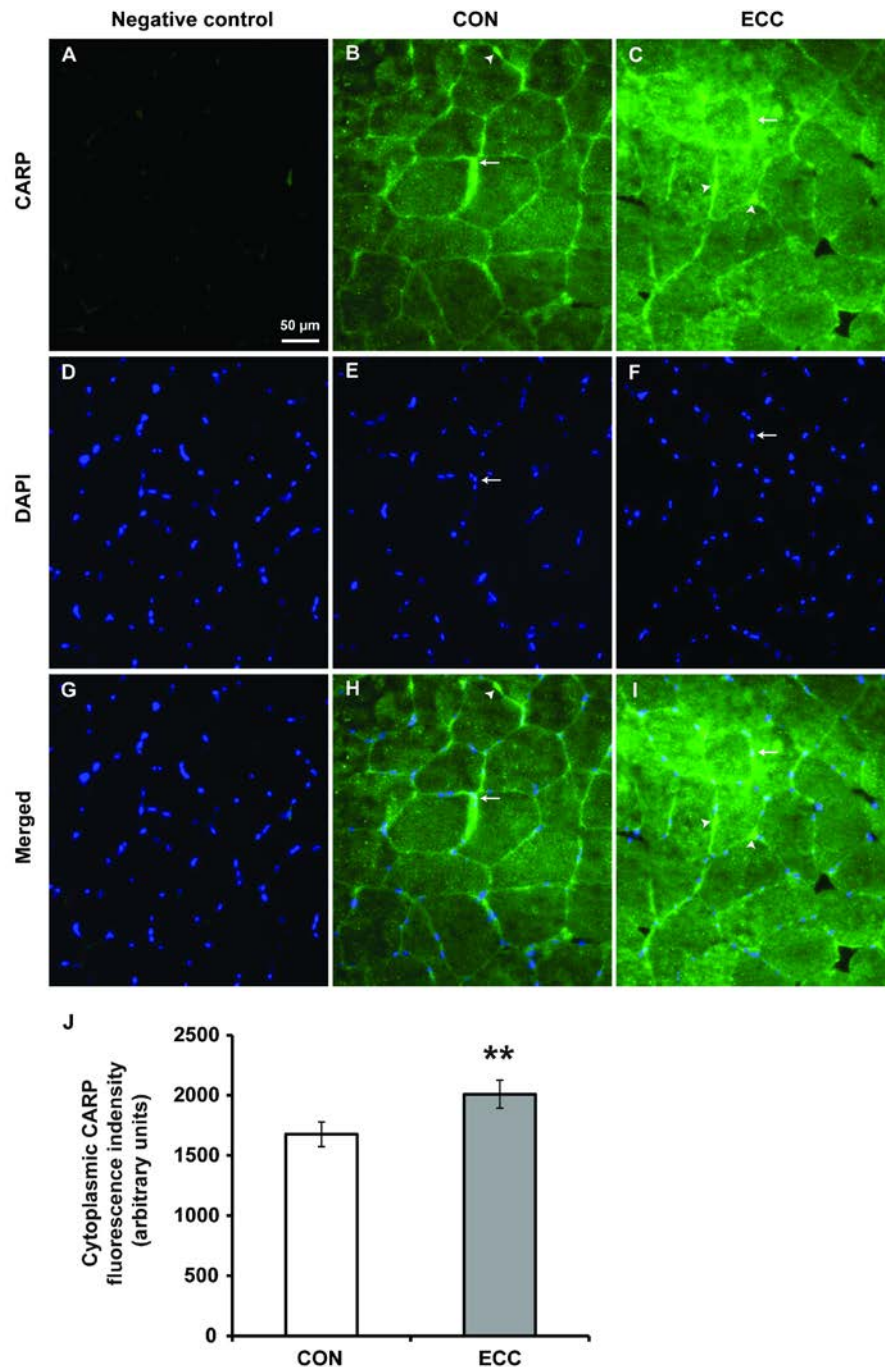


Figure 5.3. Immunofluorescence analysis of ANKRD1 protein expression level at 24 h post-eccentric exercise in the *vastus lateralis* muscle. A-I) Double-labeled immunofluorescence staining of ANKRD1 (green) and DNA (blue DAPI staining) in the negative control (without the anti-ANKRD1 primary antibody), non-exercised control (CON), and 24 h post-eccentric exercised (ECC) samples. Arrows indicate the nuclei that are positive for ANKRD1. Arrowheads indicate the ANKRD1 positively stained area with negative DAPI staining. J) Quantification of cytoplasmic ANKRD1 staining intensity with results presented as mean \pm SEM. ** $P < 0.01$ (ECC vs. CON leg).

Induction of ANKRD1 mRNA In Different Mammalian Muscle Damage Models: To determine if an elevation in *ANKRD1* is unique to eccentric exercise or a more general phenomenon, I queried the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) repository. I surveyed the datasets for muscle damage models and diseases and selected the five microarray data sets that focused on skeletal muscle disorders to analyze *ANKRD1* expression. One data set included human quadriceps samples from 11 healthy individuals and 12 DMD patients. There was a significant ($P = 0.006$) increase ($FC = 2.8$) in *ANKRD1* gene expression level in the DMD patients vs. healthy individuals (Figure 5.4A). Analysis of the second data set, which contained gastrocnemius muscles of five wild-type and five mdx mice, demonstrated that *ANKRD1* mRNA was significantly increased in the mdx vs. wild type mice ($FC = 12.3$, $P = 0.0003$, Figure 5.4A). The third data set examined the effects of acute freeze injury on mouse skeletal muscle gene expression. This insult resulted in a highly significant ($N=3$ animals per group; $P < 0.0001$) induction of *ANKRD1* gene expression in damaged versus control muscle ($FC = 47.6$). In addition, the diaphragm muscles of mice with $\alpha 2$ -laminin deficient muscular dystrophy ($N = 4$) had a significantly ($P = 0.005$) elevated levels ($FC = 6.2$) of *ANKRD1* relative to control mice ($N = 4$) (Figure 5.4A). Finally, an analysis of humans with Infantile Onset Pompe disease revealed that the bicep muscles of affected individuals ($N=9$) contained significantly higher levels of *ANKRD1* mRNA than the controls ($N=10$) ($FC = 1.8$, $P < 0.0001$) (Figure 5.4B). These data, coupled with our observations of eccentrically exercised human muscle, suggest that *ANKRD1* induction is a common feature of most muscle injury models.

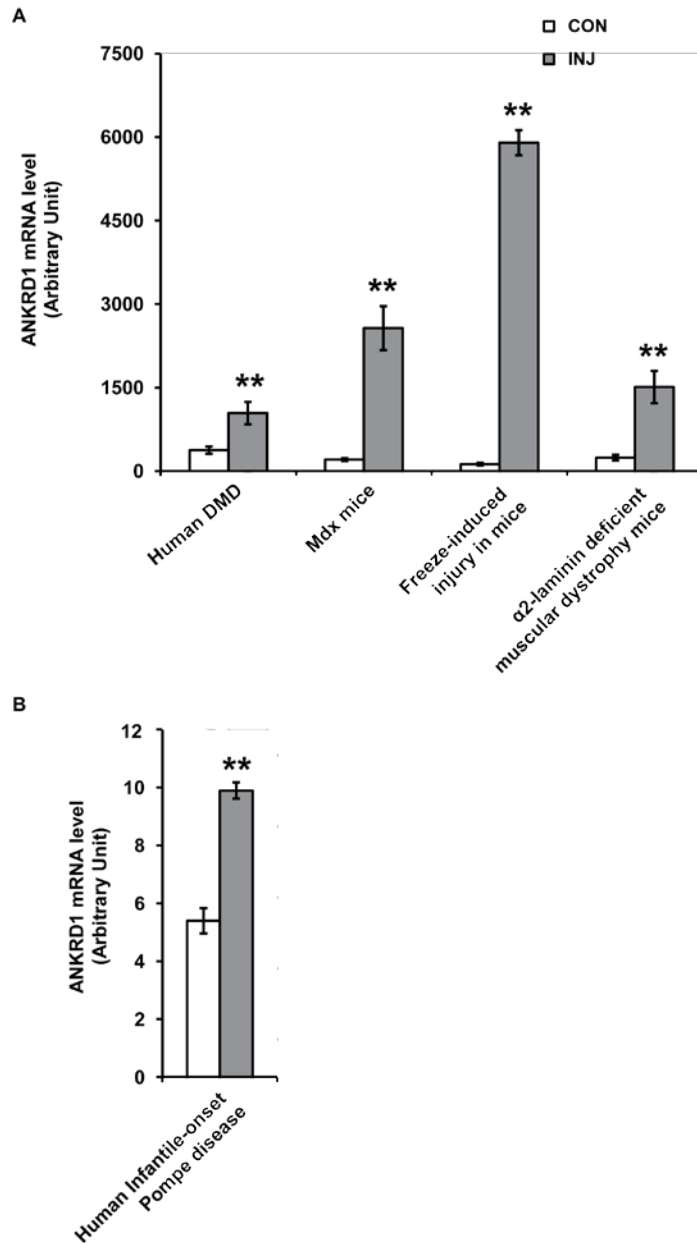


Figure 5.4. Comparison of ANKRD1 mRNA levels in the normal/wild type control (CON) vs. injured (INJ) muscle in different mammalian muscle damage models. Five data sets from the GEO microarray database were examined for ANKRD1 expression in skeletal muscle of Duchenne muscular dystrophy (DMD) patients, dystrophin-deficient mdx mice, freeze-induced muscle injured mice, α 2-laminin deficient muscular dystrophy, and humans with infantile onset Pompe disease. Data are presented as mean \pm SEM. ** $P < 0.01$ (INJ vs. CON).

Discussion

In the current study I demonstrated that ANKRD1 mRNA was induced in skeletal muscle in various muscle damage models including eccentric exercise, muscle dystrophy, and freeze-induced injury. Our data also indicated a significant increase in cytoplasmic ANKRD1 protein levels 24h post-eccentric exercise. Most importantly, for the first time, our data indicated that ANKRD1 nuclear accumulation is positively correlated with muscle strength loss (Figure 5.2). These data strengthen the previously proposed function of ANKRD1 as a stress-responsive protein that links mechanosensory signals from the cytoplasm to the genetic programming in the nucleus (72).

ANKRD1 induction in response to damaging stimuli

Based on our microarray analysis and subsequent qPCR confirmation, I determined that the ANKRD1 gene (*ANKRD1*) is highly induced in human *vastus lateralis* muscle at 3 h post-eccentric exercise. This result is consistent with previous studies that also reported increased levels of *ANKRD1* at 3h, 4-8 h, or 48 h in humans following eccentric exercise (23, 95). It is interesting to note that the level of this change was more than an order of magnitude greater in this study than in the previous reports. Chen et al. (7) examined gene expression at 4-8 h post-exercise and observed a 7.5-fold increase in *ANKRD1* expression, as compared to the 103.4 fold I observed. In a separate report, Mahoney et al. (95) observed changes in *ANKRD1* expression of 9.7 fold at 3 h post-exercise and 14 fold at 48 h post-exercise. Since several of these time points were similar to the one in our study, this discrepancy is unlikely to be due to timing. Instead, I suspect that these differences in gene expression may be due to the different modes of

exercise employed. In the current study, subjects performed 100 eccentric actions at 30°/sec on a dynamometer, which cause significant muscle damage as demonstrated by a 42.1% decrease in strength. In contrast, Chen et al. (23) had subjects complete 300 eccentric and concentric actions in the format of rising from, and lowering to, a seated position using one leg. In the study by Mahoney et al (95), the 300 eccentric actions were conducted at a much faster speed (120°/sec). While not measured directly, these exercises may induce less muscle damage than the one that I employed.

To extend our understanding of the potential involvement of ANKRD1 in muscle damage, I took advantage of the publically available GEO database. These microarray datasets represent a diverse set of unbiased measurements of gene expression in a wide variety of conditions. I selected five different models of mammalian muscle damage and analyzed ANKRD1 mRNA expression. Previous studies have reported an elevation in ANKRD1 expression in human Duchenne muscular dystrophy (DMD) (6) as well in four different models of muscle dystrophy associated with mutations or deficiencies in: calpain-3, dysferlin, α -sarcoglycan and dystrophin (78), and peroxisome proliferator-activated receptor-induced myopathy (20). Therefore, our data extend the range of muscle disorders that display an elevation of ANKRD1 expression following muscle injury and strengthen that ANKRD1 elevation is a universal response to muscle damage stimuli.

Our immunofluorescence analysis showed an increase in ANKRD1 protein in the cytoplasm at 24 h post-exercise. Given that I observed a dramatic increase in ANKRD1 mRNA at 3h post-exercise, it is not surprising that there was an accumulation of the protein at 24 h post-exercise. Consistent with our finding, several previous studies have also reported that ANKRD1 protein was increased in skeletal muscles from patients with

congenital myopathy and spinal muscular dystrophy (116), DMD and congenital muscular dystrophy(117), as well as in patients with atrophic muscles in amyotrophic lateral sclerosis (118). Taken together, the data from the current study, and previous reports, supports the hypothesis that ANKRD1 mRNA and protein increase after an a damaging insult in muscle. Furthermore, this elevation appears to persist in chronic muscle disorders like muscular dystrophy, amyotrophic lateral sclerosis, Pompe's disease, etc..

ANKRD1 translocation from cytoplasm to nucleus after eccentric exercise

It has been proposed that ANKRD1 may act as a messenger that links mechanosensory processes in the cytoplasm to transcriptional regulation in the nucleus (72), although direct tests of this hypothesis are limited. To help evaluate this hypothesis, I fractionated control and eccentrically exercised human skeletal muscle into cytoplasmic and nuclear fractions and assessed the subcellular localization of ANKRD1 via Western blotting. I observed clear nuclear accumulation of ANKRD1 protein in a subset of the eccentric-exercised samples (Figure 5.2A). The failure of these data to achieve statistical significance is likely due to the small sample size (N = 7) and high inter-subject variations that is common for human studies. I speculate that with more subjects or the utilization of appropriate animal models, that the nuclear translocation of ANKRD1 post-exercise would be more robust. Indeed, one mouse study by Tsukamoto et al. (163) provided indirect evidence supporting the possibility of ANKRD1 nuclear translocation post-muscle damage. In that study they used immunohistochemistry to monitor the translocation of the ANKRD1 ortholog, ANKRD2, from sarcomeric I-band to the nucleus

in gastrocnemius muscles of mice after injury induced by cardiotoxin injection or freeze injury (163). Since ANKRD2 and ANKRD1 share ~50% identity at the amino acid level, as well as structural organizations that include several common domains such as four ankyrin repeats and an N-terminal nuclear localization signal (NLS) (106), it is likely that ANKRD1 and ANKRD2 have overlapping function. The possibility that ANKRD1 translocations to the nucleus is supported by our Western blot data which showed the nuclear accumulation of ANKRD1 post-eccentric exercise in subject #2, #4, and #7 (Figure 5.2A).

ANKRD1 is a potential biomarker for skeletal muscle damage

I found that the nuclear accumulation of ANKRD1 was positively correlated ($P = 0.007$) with the peak muscle strength loss in the 24h-Study. This is the first study to address the relationship between nuclear ANKRD1 protein content and an indirect marker of EIMD. Therefore, nuclear ANKRD1 content could represent an alternative biomarker for EIMD. Considering that ANKRD1 induction is observed in a large number of muscle damage models and diseases, ANKRD1 might serve as a universal biomarker for muscle damage.

Upstream regulators and downstream target genes of ANKRD1 after eccentric exercise

Our data demonstrated that ANKRD1 was induced after eccentric exercise and may translocate to nucleus. Of interest are the signal transduction pathways that drive ANKRD1 transcription. In cardiomyocytes, transcription factor GATA4 regulates

ANKRD1 gene expression via binding response elements in the *ANKRD1* promoter (93). In vascular smooth muscle cells, transforming growth factor- β 1 (TGF- β 1) induces ANKRD1 mRNA via its downstream mediator Smad3, which binds to the CAGA element in the *ANKRD1* promoter (67). In addition, IL-1 α and TNF- α increase *ANKRD1* expression in human endothelial cells (25). NF- κ B has been shown to bind to the *ANKRD1* promoter and upregulate *ANKRD1* in a model of human airway smooth muscle cells in which the desmin gene was knocked down (108). Indeed, two studies support a role for NF- κ B in the induction of ANKRD1 gene expression (57, 109) in skeletal muscle. For example, I have previously reported (57) that NF- κ B activity is increased 3 h post-eccentric exercise in the same muscle biopsy samples that were employed for this study. Furthermore, Mohamed et al. (109) demonstrated that skeletal muscle longitudinal stretch-activated NF- κ B induces *ANKRD2* gene expression with a promoter-reporter system. Given the high similarities between ANKRD1 and ANKRD2, it is possible that ANKRD1 would be induced via stretch-induced injury.

ANKRD1 functions as a transcriptional cofactor that can positively or negatively regulate the expression of target genes (3, 62, 71, 176). In cardiomyocytes, ANKRD1 has been characterized as a negative transcription cofactor that binds to YB-1 and suppresses the expression of myosin light chain-2v, troponin C, and atrial natriuretic factor gene expression (62, 176). A recent study also reported that ANKRD1 repressed MMP14 and MMP10 gene transcription in skin fibroblasts (3). In contrast to these examples of gene repression, Kojic et al. (71) demonstrated that ANKRD1 can behave as a transcription co-activator of p53, and thus positively influence the expression of p53 target genes,

including *P21*, *MDM2*, and *ANKRD2*. It will be valuable to define the mechanisms by which ANKRD1 influences gene expression during eccentric exercise.

Proposed ANKRD1 action model after eccentric exercise

In Figure 5.5 I propose a model for how ANKRD1 expression may be regulated following damaging eccentric exercise. Under resting conditions, there is some nuclear ANKRD1, but the majority of the ANKRD1 protein is localized predominantly in the cytoplasm where it binds to titin at the sarcomeric I-band (72) (Figure 5.5A). During and/or shortly after eccentric exercise (Figure 5.5B), the mechanical damage from the eccentric exercise induces a stress response that includes the activation and translocation of NF- κ B into the nucleus where it binds to the NF- κ B binding site located in the ANKRD1 gene promoter and thus induces ANKRD1 expression (denoted with the “+”). ANKRD1 mRNAs is then translated in the cytoplasm where it accumulates. Meanwhile, the mechanosensory complex that includes the ANKRD1 protein senses the mechanical stretch from the exercise and ANKRD1 translocates into the nucleus. As a transcriptional regulator, ANKRD1 binds to YB-1 and perhaps other transcription factors that then act to enhance or repress the expression of subordinate genes, which ultimately contribute to muscle remodeling and /or regeneration.

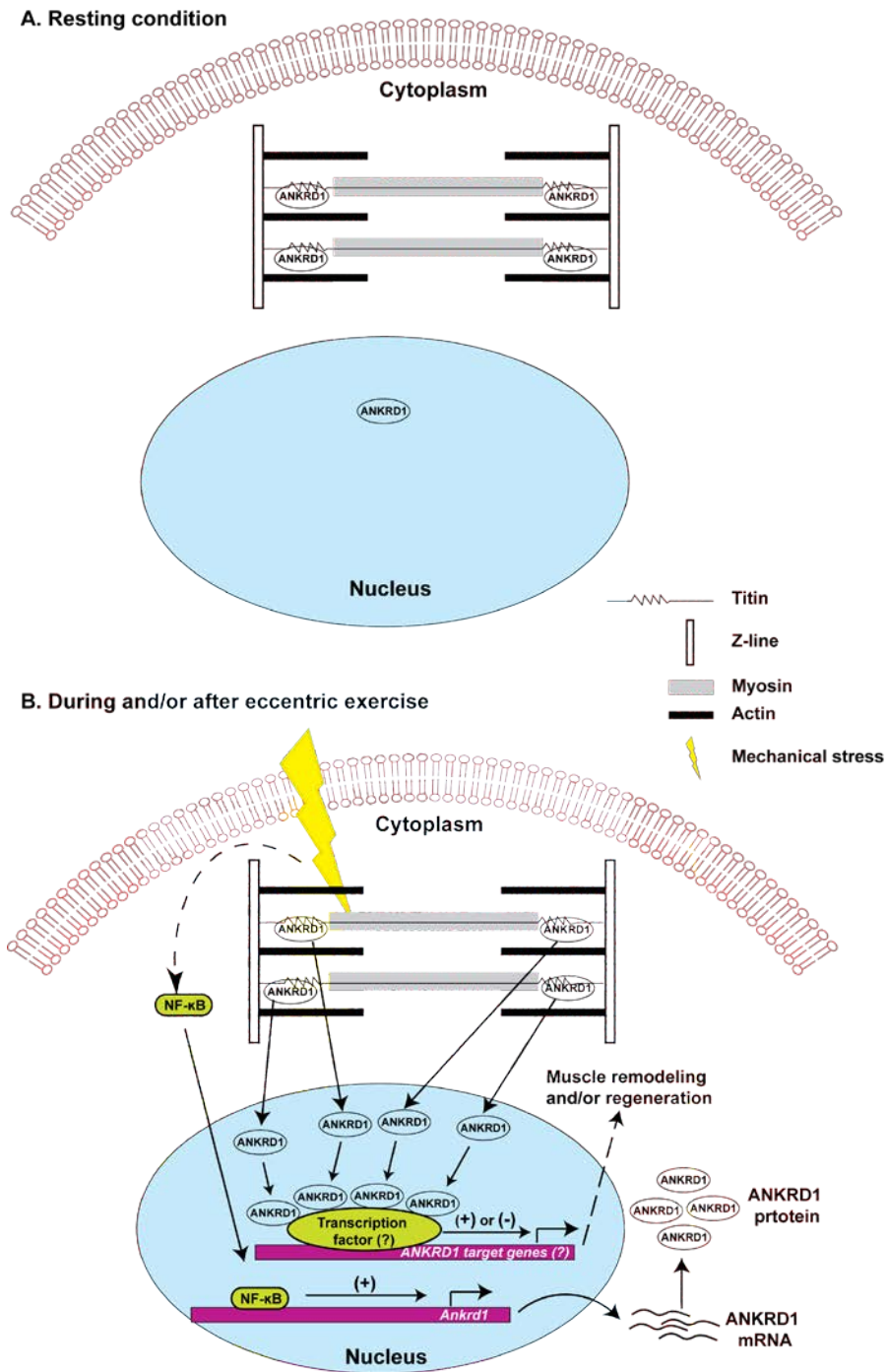


Figure 5.5. Model of ANKRD1 responses in skeletal muscle following eccentric exercise. A. At rest. B. During and/or following damaging eccentric exercise. Question marks indicate unknown molecules; solid arrows indicate known process and dashed arrows demonstrate unknown signaling cascade. See detailed explanation in the texts.

Conclusions

Our data provides evidence of the induction of ANKRD1 mRNA and protein following eccentric exercise in human skeletal muscles. I also report on microarray studies that suggest that an induction of ANKRD1 expression may be a common feature of acute and chronic skeletal muscle injury. Most importantly, these data provide the first demonstration that nuclear ANKRD1 accumulation is positively correlated with muscle strength loss following damaging eccentric exercise. Collectively, these data strengthen the possibility of ANKRD1 as a universal marker for skeletal muscle damage and further implicate that ANKRD1 acts as a link between mechanosensory signaling and gene reprogramming which contributes to muscle remodeling/regeneration after eccentric exercise.

CHAPTER 6

SUMMARY AND FUTURE DIRECTIONS

Summary

Unaccustomed, strenuous and eccentric exercise results in temporary muscle damage which is partially due to an excessive inflammatory response. A bout of intense eccentric exercise leads to adaptation in that muscle so that the same muscle is more resistant to the damage induced by a subsequent similar exercise bout, a phenomenon known as repeated bout effect (RBE) (53, 55, 100, 126, 132, 133). There are conflicting data (30, 48, 54, 103, 122, 155) regarding the presence of the CRBE, whereby exercising one muscle group induces an adaptation to the contralateral muscle group that results in an attenuated damage response when it is subjected to the same exercise at a later time. Since the CRBE has received only modest attention, little is known about the mechanisms for this phenomenon. **The first aim** of this dissertation was to determine if the CRBE exists in leg muscles and to determine if the activity of the NF- κ B pathway, a key inflammatory regulator, is altered during the CRBE. In Study I (Chapter 3) of this dissertation, I had observed that when subjects performed one bout of unaccustomed eccentric exercise in one leg, the other leg experienced less muscle damage five weeks later when it is exposed to the same exercise. Furthermore, I found that the DNA-binding activity of NF- κ B was lower than anticipated in the second exercise bout in the contralateral leg. In Study II (Chapter 4), I assessed the activation level of an upstream regulator of NF- κ B (I κ B α) and the expression of some downstream NF- κ B-associated inflammatory genes in the biopsy samples collected from the subjects who performed two

exercise bouts in opposite legs. Results from Study II showed that the phosphorylation of I κ B α did not change significantly either after the first exercise or between the two bouts of exercise. The failure to detect changes in I κ B α phosphorylation could mean that it is not a central regulator in the NF- κ B pathway in this system or that there were technical issues that precluded seeing the underlying change, such as selecting the wrong time point for analysis (3 h post-exercise). One of the key results of my study was the demonstration that the expression of three NF- κ B-associated inflammatory genes (*ANKRD1*, *IL1R1*, and *CYR61*) a blunted increase in was attenuated in bout 2 versus bout 1 of exercise. These data from Study I and II support our hypothesis that the CRBE may be partially due to an attenuated inflammatory response mediated by NF- κ B signaling pathway.

In Study II, I used microarray and qRT-PCR analysis to examine the CRBE and observed that ANKRD1 was the most dramatically induced gene following eccentric exercise. Therefore, Study III (Chapter 5) investigated **the second aim of this dissertation** to determine if ANKRD1 is a generalized marker of skeletal muscle damage induced by eccentric exercise or disease. I found that ANKRD1 mRNA was dramatically increased in a variety skeletal muscle damage models. I also found a positive correlation between ANKRD1 protein levels in human skeletal muscle following eccentric exercise and muscle strength loss, further supporting the hypothesis that ANKRD1 is a muscle damage biomarker.

In conclusion, the data presented in this dissertation confirms the existence of the CRBE and provides regulatory insights into the underlying molecular mechanisms. In addition to expanding our understanding of this phenomenon, these findings may be of

potential importance for setting up rehabilitation exercise or identifying therapies to facilitate muscle recovery from injury. For example, if one leg of a patient was immobilized due to an injury or a disease, eccentric exercise of the healthy leg before rehabilitation begins for the immobilized limb may provide protection against exercise-induced muscle damage for the immobilized leg during rehabilitation exercise, thereby facilitating recovery from injury. In addition, data from this dissertation suggests that an induction of ANKRD1 expression may be a universal feature of acute and chronic skeletal muscle injury that may have some utility as a diagnostic marker.

Future Directions

Data presented in Study I support the existence of the CRBE in healthy young men. Although the CRBE has potential clinical relevance, the investigation of this phenomenon is still in an early stage. Only a few studies (30, 48, 54, 103, 122, 155) have investigated the existence of the CRBE with equivocal results. There are still a number of general questions that need to be answered about the CRBE. For example, is the CRBE gender and age specific. Also, we do not yet know how long the CRBE lasts. We must also identify the minimal amount of damaging exercise in regard to intensity and volume that must be completed to induce a CRBE. It is also imperative to define if the CRBE occurs with different damaging modalities (exercise with voluntary effort or electrical stimulation of muscles). At the cellular and molecular levels, key questions include: are there humeral or neuronal factors that mediate this phenomenon; and are all of the molecular responses to damaging exercise attenuated or only a subset. To answer the first set of questions, future studies will need to better characterize the existence and

magnitude of the CRBE within various populations (e.g., women and elderly people), with different exercise modes, intensity, speed, or different time intervals between the two exercise bouts. Additional physiological studies will be required to determine the nature of the signaling events that couple the first bout of exercise to the attenuation of responses in the contralateral muscle following the second bout.

In study I and II of this dissertation I used muscle biopsy samples collected at 3 h post-eccentric exercise and observed a blunted increase in nuclear NF- κ B DNA-binding activity and an attenuated upregulation of three NF- κ B-related inflammatory genes (*ANKRD1*, *CRY61*, and *IL1R1*) in bout 2 compared to bout 1. It should be noted that skeletal muscle contains many different types of cells including myofibers, satellite cells, red blood cells, pericytes, and resident inflammatory cells. Therefore, it is not definitively known if the observed changes in gene expression occurred in myofibers or other cell type(s). It will be important to perform anatomical studies to determine the relative changes in gene expression in each of these cell types. Indeed, our laboratory has previously demonstrated that the activation of NF- κ B 3 h post-eccentric exercise is primarily localized to skeletal muscle-residing pericytes (57). Additional studies using immunofluorescence and *in situ* hybridization methods are needed to localize the attenuated NF- κ B and less increase in the inflammatory genes in bout 2 compared to bout 1.

It should be noted that the contralateral leg was not exposed to exercise in the initial bout and thus there was no direct stimulus for molecular and cellular adaptation such as reduced NF- κ B activation or blunted inflammatory genes in the contralateral leg. Therefore, the attenuated NF- κ B mediated inflammatory response may not be a driving

or independent mechanism for the CRBE. Theoretically, the potential sites that may be involved in a CRBE include muscle, spinal cord or cortex. To date, only two studies (52, 155) have suggested that neural adaptation is critical for the CRBE. More studies are required to determine neural and muscular mechanisms involved in the CRBE, especially to identify neural and humoral factors and signaling pathways that contribute to the CRBE.

In summary, the CRBE is an intriguing feature of muscle response to damaging exercise that may allow the individual to more quickly adapt to activity. Defining the signaling mechanisms may provide new tools for improving fitness and thus health and the quality of life.

APPENDIX

ORIGINAL STUDY DESIGN

This study was originally intended to examine the effects of two botanical supplements containing antioxidant and anti-inflammatory components on gene expression (transcriptome patterns) via microarray analysis and muscle functional changes in response to eccentric exercise in humans. The sample size for a paired analysis (same subject measured pre and post) is generally less than for a non-paired analysis (two sets of subjects) since the gene expression variation is reduced in the paired design. A paired design with a gene level standard deviation of 0.4 on the log scale has ~80% power at an α level of 0.005 to detect a fold change of 1.5 with ~10 subjects. In a double-blind design, 31 healthy men (18-30 yrs) were randomly assigned to receive the placebo (N=11), Supplement 1 (N=10), or Supplement 2 (N=10) for 35 d. Subjects performed two bouts of eccentric exercise spaced 4 wks apart (Figure below). On Visit 0, subjects were randomly assigned into one of 3 groups: Placebo, Supplement 1, or Supplement 2. Bout 1 was pre-supplement period and was composed of 7 visits. The details of the visits are provided in Chapter 3, Study I. At the end of bout 1, subjects started taking supplements or placebo for 28 d and continued taking it during bout 2 period (7 days) for a total of 35 d of supplementation.

Supplement 1 and Supplement 2 were botanical formulations, each consisting of antioxidant, anti-inflammatory, properties designed to reduce oxidative stress and inflammation (see Table below). All treatments were provided in tablets identical in size, shape, and color and supplied in bottles containing a 7-day supply. On each day during supplementation period, subjects took 3 tablets in the morning with breakfast. Subjects

received a 7-day supply of treatment each week for five weeks. Upon their weekly visit to receive another 7-day supply, they returned the empty pill containers or containers with unused supplements. Supplement accountability was noted by the investigator during these visits.

Using a repeated measures ANOVA, I found no significant differences between the supplement and placebo groups (no significant group or group \times bout interaction) for CK, muscle soreness, or strength. Because I did find a significant bout effect for muscle strength, I pooled the data from all subjects in to further examine the contralateral Bout effect and potential underlying molecular mechanisms in muscle, which formed the basis of this dissertation proposal.

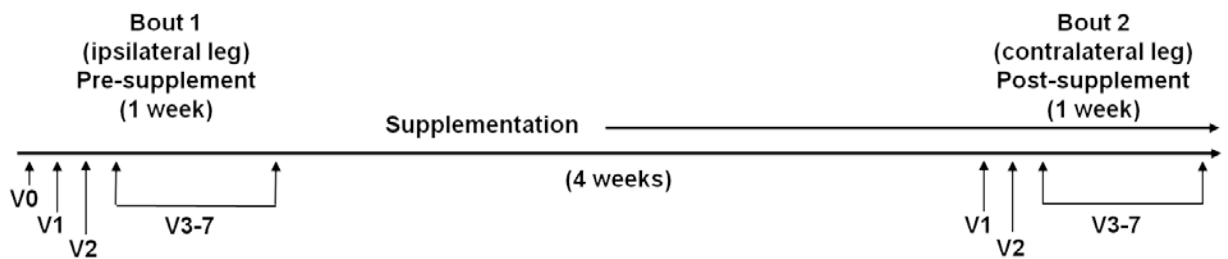


Figure A.1. Study design. Visit 0 (V0): informed consent and interview; Visit 1 (V1): blood draw, muscle strength tests; Visit 2 (V2): muscle soreness evaluation, pre-exercise muscle strength tests, eccentric exercise, post-exercise muscle strength tests, muscle biopsies 3 h post-exercise; Visits 3 - 7 (V3-7): blood draw, muscle soreness evaluation, muscle strength tests.

Table A.1. Supplement treatment

| Treatment | Schedule: 35 days |
|--|---|
| Supplement 1 600 mg rose hips + 200 mg of rhodiola + 4 mg astaxanthin per dose | 3 tablets (corresponding to 1 dose) in the morning with breakfast |
| Supplement 2 125 mg ashwaganda + 500 mg of prickly pear + 200 mg of grape seed extract per dose | 3 tablets (corresponding to 1 dose) in the morning with breakfast |
| Placebo microcrystalline cellulose, dextrose, modified corn starch, modified cellulose gum, stearic acid, and silicon dioxide per dose | 3 tablets (corresponding to 1 dose) in the morning with breakfast |

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