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**MOLECULAR MECHANISMS UNDERLYING THE MUSCLE RESPONSE TO
EXERCISE-INDUCED MUSCLE DAMAGE IN CHRONIC CIGARETTE SMOKERS**

A Dissertation Presented

by

NINA A. MOORE

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

DOCTOR OF PHILOSOPHY

September 2015

Kinesiology

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A Dissertation Presented

by

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DEDICATION

I dedicate this dissertation in part to my family—my parents John and Cass, my future husband Jon, and sweet little nuggets Noah, Lily, and Kody. I could not have done this without you, and am so lucky and thankful for your love and support.

Foremost, I dedicate this dissertation to the memory of my former advisor, mentor, and friend, Priscilla M. Clarkson. Thank you for taking in this little stray.

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ABSTRACT

MOLECULAR MECHANISMS UNDERLYING THE MUSCLE RESPONSE TO EXERCISE-INDUCED MUSCLE DAMAGE IN CHRONIC CIGARETTE SMOKERS

SEPTEMBER 2015

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Smoking increases risk for musculoskeletal injury and protracts healing. The underlying mechanisms have not been explored, yet dysregulated inflammatory responses may contribute. To investigate these risks, Study 1 of this dissertation monitored typical responses to muscle damaging eccentric contractions (ECC) in smokers and non-smokers. Smokers experienced greater muscle fatigue both during and immediately after ECC and greater delayed hyperalgesia, a phenomenon known as delayed onset muscle soreness (DOMS), than did non-smokers. DOMS (driven by inflammation) peaks 48h after ECC, when there is a shift from pro- to anti-inflammatory signaling; thus I chose to investigate this time further in smokers. The “late inflammation” time point is relatively unstudied mechanistically. Therefore, in Study 2 I further characterized the post-ECC muscle response in non-smokers and focused on hyperalgesic pathways. Transcriptome analyses revealed greater proliferation and pro-inflammatory signaling at 3h, which returned to baseline by 48h. Activity of key regulatory pathways canonical (p65) nuclear factor kappa-beta (NFκB) and extracellular regulated kinase (ERK)1/2 mimicked the pro- (activated) to anti-

inflammatory (de-activated) shift. In addition, nerve growth factor (NGF), a hyperalgesic-modifier, was also induced. Further testing revealed suppressed 48h p65 activity. In Study 3 I tested these pathways, and non-canonical NFκB (REL-B) signaling (specifies oxidative muscle fibers), in skeletal muscle from smokers and non-smokers 48h post-ECC. Smokers had fewer oxidative (Type I) muscle fibers. Further, REL-B, enhanced in non-smokers at 48h, was suppressed in smokers. These findings may explain the greater fatigue in smokers, which can increase risk for injury. p65 activity, suppressed at 48h in non-smokers, was unchanged in smokers, suggesting delayed pro-inflammatory resolution. Active and total ERK levels were suppressed, overall, in smokers. While there were no differences in NGF levels or co-localization with the TrkA receptor between smokers and non-smokers, smokers did express higher p75^{NTR} receptor levels, which may activate NFκB and promote hyperalgesia. NGF/p75^{NTR} co-localization at 48h post-ECC decreased in non-smokers but increased in smokers. Higher DOMS, NGF, and NFκB signaling may indicate prolonged pro-inflammatory signaling, delayed resolution, and protracted healing in smokers. This dissertation provides insights into mechanisms that appear to place smokers at greater risk for musculoskeletal injury and impaired healing.

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CHAPTER I

INTRODUCTION

Statement of the Problem

Cigarette smoking is associated with increased risk for musculoskeletal injury (145, 157) and prolonged healing (5, 182). One common factor underlying many of the negative health consequences of smoking is a dysregulated inflammatory environment (23, 71, 81, 101, 320, 329, 348), which could contribute to increased musculoskeletal injury risk and delayed recovery. However, the precise molecular mechanisms linking smoking to increased injury risk are currently unknown.

Muscle is a plastic tissue that undergoes constant change, either through normal protein turnover or, when exposed to exercise, by undergoing a highly coordinated program of remodeling. Remodeling can change muscle at structural and molecular levels, such as hypertrophy and fiber type shift, and consequently alters future function. When muscle is injured via strenuous exercise (especially muscle lengthening or eccentric exercise (ECC)), primary mechanical damage evokes a pro-inflammatory response, which can then result in secondary damage. Secondary damage increases muscle breakdown, yet also stimulates repair and regeneration of the muscle. Repeated exposure to ECC results in adaptation, through which the muscle is strengthened and protected against future insult. Without secondary damage and inflammation, regeneration is delayed (190, 221) and adaptation is attenuated (168, 223).

Secondary damage from inflammation can be categorized into two phases: early (~ 0 -2d after the initial insult) and late (≥ 2 d), which can overlap around the 2d post-exercise time point. Under normal conditions, there is a balance between pro- and anti-inflammatory processes needed to maintain muscle homeostasis. In response to muscle injury, specifically exercise-induced muscle damage (EIMD), the inflammatory balance tips to first favor pro-inflammatory actions (early phase), then anti-inflammatory ones (late phase), finally ending in resolution back to baseline. The shift between these phases, which occurs approximately 2d post-exercise, is an important time point in muscle regeneration. The majority of research into EIMD has focused on the gross responses to ECC such as strength loss, while fewer studies have examined molecular responses, mostly at early (< 1 d) time points. The early phase of secondary damage has been moderately well explored (137, 214, 267, 313, 323), while later time points (particularly around the 2d inflammatory shifting point) remain largely unexplored. Understanding how inflammation successfully resolves after EIMD would help clinicians optimize healing.

Smokers exhibit alterations to the immune and inflammatory systems and are more susceptible to infection and complications following injury or surgery (143, 225). In smokers, cytokines and chemokines tend to promote pro-inflammatory signaling and neutrophil and macrophage numbers are enhanced with conversely suppressed activity including phagocytosis, respiratory bursts, and cytokine release (17, 23, 305, 320). Together, these alterations generally promote a more pro-inflammatory environment in smokers. However, it is unknown how this

dysregulated inflammatory environment could impact smokers' response to EIMD. It is possible that dysfunctional inflammation and disrupted molecular signaling impair myogenesis, leading to inadequate muscle repair in smokers, which may explain the increased risk for musculoskeletal injury.

Aims and Hypotheses

The **primary goal of this dissertation** was to understand how smoking affects muscle damage and recovery. To do this, I used ECC as a safe and effective model of transient muscle injury that elicits delayed onset muscle soreness (DOMS), strength loss, and the release of muscle-specific proteins into the blood. The following set of experiments was designed to test the effects of smoking on EIMD, from the functional level down to the molecular level.

Study I: Smoking Decreases Muscle Function During Eccentric Exercise and Increases Delayed Onset Muscle Soreness

The **first goal of this dissertation** was to determine if the functional responses of smokers following ECC differed from that of non-smokers. I hypothesized that, compared to non-smokers, smokers would have a more pronounced damage response, experiencing: 1) greater early strength loss; 2) more prolonged strength loss; 3) greater creatine kinase release; and 4) greater soreness (both peak and prolonged) in response to ECC.

Smokers in this study experienced greater muscle fatigue, as indicated by strength loss during (10% higher strength loss in smokers during final exercise set)

and shortly (5min post) after ECC (~15% higher strength loss in smokers). Smokers performed the same amount of work (but generated fewer high-force contractions during exercise), yet experienced the same level of prolonged (1-5d post) strength loss as compared to non-smokers. Finally, smokers experienced a higher peak (~15% greater in smokers) and greater overall (all time points combined) soreness than non-smokers after exercise. These results add to the existing body of knowledge on the consequences of smoking by showing greater early fatigue, greater soreness, and similar strength loss in response to fewer high-force eccentric contractions—indicating increased susceptibility to EIMD in smokers.

Study II: Transcriptome Analysis of Skeletal Muscle at Early (3h) and Late (48h) Time Points after Eccentric Exercise

In Study 1, I found that smokers have greater peak and overall DOMS post-exercise as compared to non-smokers. The next step was, therefore, to examine potential molecular mechanisms that could drive the differences seen in Study 1. Inflammation is widely considered the driving force behind DOMS. Given the additional importance of inflammation to the muscle repair/regeneration program, understanding mechanistic changes underlying the associated inflammation and its resolution following EIMD is critical.

Mechanisms underlying inflammatory changes in the days following ECC are poorly understood, therefore it was important to next generate an in depth description of early (3h) versus late (48h) time points post-ECC in non-smokers – to have a normal pattern of response to which I could compare smokers in a follow-up

study. Consequently, the **second goal of this dissertation** was to use skeletal muscle transcriptome data to identify molecular changes across early and late inflammatory phases after ECC, and to use these results to identify candidate genes and pathways important to EIMD inflammatory processes, especially those related to DOMS, in non-smokers. This would generate a working model of “normal” EIMD, repair, and DOMS to which I could then compare to the responses of smokers in Study 3. I hypothesized that: 1) early pathways in skeletal muscle would involve stress-response genes and immediate pro-inflammatory pathways; 2) later (at 48h), inflammation would continue to be a dominant force in the ECC response, evolving from the pro-inflammatory expression at early time points to anti-inflammatory signaling at 48h; and 3) altered expression in other signaling pathways would become evident at the later time points, including vascularization/angiogenesis and muscle structure.

Study 2 identified 3h activation of the extracellular regulated kinase (ERK)1/2 pathway that was suppressed by 48h post-ECC. The ERK1/2 pathway affects inflammatory signaling and, more specifically, promotes proliferation. I further determined that the canonical (p65: REL-A, v-rel avian reticuloendotheliosis viral oncogene homolog A) nuclear factor kappa-beta (NFκB) pathway is also altered during the later damage response, with similar activation patterns as I found for ERK1/2. The canonical NFκB pathway promotes (among other effects) pro-inflammatory actions and proliferation, while the non-canonical (REL-B, v-rel avian reticuloendotheliosis viral oncogene homolog B) pathway promotes oxidative metabolism and may affect anti-inflammatory signaling. Finally, I identified changes

over time for the co-localization of nerve growth factor (NGF) and its receptors: tyrosine receptor kinase A (TrkA, high binding affinity) and p75 neurotrophin receptor (p75^{NTR}, low binding affinity). NGF stimulates activity of the NFκB and ERK pathways, which in turn can drive NGF expression. Evidence indicates that NGF binding to its receptors is related to various types of hyperalgesia, including that induced by ECC. Overall, the findings from Study 2 add to current understanding of the EIMD response by characterizing the molecular responses at 48h post-exercise in healthy non-smokers. Having established this “normal” pattern of response, I then sought to compare molecular changes in smokers to this established pattern (Study 3), particularly at the later time point.

Study III: Molecular Effects of Smoking on Skeletal Muscle

48h after Eccentric Exercise

In Study 2, I found that the activity of ERK1/2 and canonical NFκB pathways, as well as the hyperalgesic mediator NGF, differed between early and late phases of EIMD. Components of these proposed pathways are altered in various cells/tissues in smokers (6, 53, 56, 138, 332, 368), although their expression or activity in skeletal muscle remains unexplored. The **final goal of this dissertation** was to evaluate the molecular pathways identified in Study 2 in smokers at 48h after ECC in newly collected skeletal muscle biopsies. I hypothesized that: 1) smokers would have a greater proportion of Type II fibers and suppressed REL-B activity at 48h post-ECC compared to non-smokers; 2) after ECC the activity of the canonical NFκB pathway would be suppressed in non-smokers but not in smokers; 3) nuclear

activity of the ERK pathway would increase for non-smokers while activity would be concentrated in the cytoplasm for smokers; and 4) NGF levels and receptor co-localization would be more pronounced in smokers versus non-smokers.

I found that non-smokers had an equal ratio of Type I:Type II muscle fibers and increased activation of the non-canonical (REL-B) NF κ B pathway after ECC. In contrast, smokers had a greater percentage of Type II (non-oxidative) muscle fibers and no change to REL-B activity. The REL-B pathway stimulates oxidative metabolism, and indicates a signaling mechanism through which smoking may affect muscle fiber type, resulting in lower fatigue resistance and higher risk for injury in smokers.

p65 activity, suppressed post-ECC in non-smokers, did not change in smokers and thus could impede inflammatory resolution. Smokers had suppressed ERK activity in the cytosol and, in the nucleus, a diminished capacity for ERK activity due to lower total ERK, suggesting that smokers may have a blunted ERK response when a more substantial one is needed. NGF levels did not differ with exercise or smoking and, although smokers had greater TrkA expression, NGF/TrkA co-localization was not affected by smoking or exercise. p75^{NTR} protein expression was elevated in smokers but not with exercise. NGF/p75^{NTR} co-localization was greater than NGF/TrkA co-localization in both groups. Further, NGF/p75^{NTR} co-localization was greater overall in smokers and, after ECC, decreased in non-smokers but was enhanced in smokers. Altogether, these data suggest a mechanism through which smoking may prevent the resolution of NF κ B activity, promoting further pro-inflammatory activity, and enhance DOMS after EIMD.

The findings from Study 3 add to our current understanding of muscle health and the EIMD response at 48h post-exercise in smokers. Further, they suggest mechanisms through which smoking leads to suppressed fatigue resistance during ECC and greater DOMS. Together, these data offer several means through which smoking could enhance susceptibility to injury.

Significance

Smoking is still common in many parts of the world, and rates are higher among various groups including active duty military personnel. Smokers have a higher risk for musculoskeletal injury and disability, leading to loss of work and combat time, increased risk for future injury, and a high financial burden associated with loss of productivity and medical costs. Therefore, these data are of significant interest to public health. This line of research helps us understand why smokers are more susceptible to the negative effects of muscle stress. I have shown that smokers experience higher soreness (which could make smokers less prone to continue an exercise program), indicating a dysregulated inflammatory program post-exercise, and suggest NGF, via the p75^{NTR} receptor, is a mechanism to explain the greater soreness.

Data from these studies may help explain increased risk for musculoskeletal injury in smokers, as well as provide insight into prolonged healing in smokers (5, 143, 157, 317). In addition, I have added to our knowledge of the normal role of inflammatory signaling in muscle damage and remodeling through NFκB and NGF signaling. These data further inform the field about the signaling shifts that occur at

48h post-exercise, and how alterations to this shift may ultimately affect muscle repair and overall muscle health.

CHAPTER II

LITERATURE REVIEW

Introduction

Cigarette smoking is associated with a wide variety of negative health consequences, from increased susceptibility to viral and bacterial infection (64, 153, 253) to diseases such as chronic obstructive pulmonary disease (COPD) and an array of cancers (106, 301). Less well characterized are the effects of chronic smoking on skeletal muscle. Epidemiological studies report increased risk of musculoskeletal injury and prolonged disability in smokers (5, 145, 182), though the cellular and molecular mechanisms driving these associations are not clear.

Skeletal muscle is a plastic and resilient tissue that can adapt to a variety of conditions, including alterations to activity (15, 142, 323, 380, 381). Strenuous (typically novel) activity can damage muscle, which then undergoes repair during which irreparable tissue is removed, muscle progenitor cells fuse with surviving fibers, and new proteins are produced and incorporated into the contractile apparatus. Successful repair is often, but not always, complemented by muscle adaptation via the remodeling of existing fibers (380, 383), resulting in attenuated future damage responses to similar stresses (77).

Inflammation is vitally important in muscle repair and remodeling and changes in the inflammatory process are a potential mechanism by which smoking could increase injury risk. Both pro- and anti-inflammatory processes take place following the damage-inducing stimuli in a time-dependent manner, first to generally enhance, and later to quench inflammation and promote proliferation and

healing. From 0d to 1-2d following a damaging stimulus, the inflammatory environment is predominantly pro-inflammatory, referred to throughout this dissertation as the “early” phase of inflammation. A large pro-inflammatory response can create secondary damage, especially if unquenched. Quenching occurs as the muscle shifts into a second, anti-inflammatory phase of inflammation (referred to throughout this dissertation as the “late” phase of inflammation), which also serves to stimulate repair and adaptation. The transition from early to late phase occurs ~2d after damage, though timing can vary depending on the stimulus and high inter-subject variability. Without proper shifting of the inflammatory balance during recovery, repair can be delayed and adaptation attenuated (168, 221, 223, 342). However, the mechanisms controlling damage and repair during this shift are poorly understood.

Exposure to cigarette smoke can alter inflammation, affecting damage and repair responses. Higher rates of secondary complications after injury and surgery (infection, attenuated healing, longer time to recovery (163, 182, 316, 317)) occur in smokers, effects thought to be partially due to dysregulated inflammation (32, 101, 165, 305, 306, 316, 320). Recent experimental data showed that smoking suppressed inflammation and wound healing one week after a skin punch biopsy injury, while smoking cessation promoted a return to normal inflammatory responses and wound healing (317). With previous data regarding smoking and disability after musculoskeletal injury, these results suggest that dysregulated inflammation in smokers could be a possible mechanism through which smoking impairs healing, promotes disability, and increases the risk for future injury.

The **primary goal of this dissertation** was to understand how smoking affects muscle damage and recovery. I first investigated the effects of smoking on skeletal muscle function and markers of damage following exercise. Second, I explored in-depth the molecular processes that occur during the shift from “early” to “late” phases of inflammation after exercise to provide better context for understanding the final portion of my dissertation, which hypothesized that dysregulated inflammation during damage/repair in smokers causes greater damage and inhibits muscle repair. The following literature review provides a summary and discussion of smoking and injury risk, normal muscle damage and repair, and possible mechanisms by which smoking could modify damage and repair.

Cigarette Smoking

Cigarette smoking has been associated with a variety of adverse health effects ranging from inflammatory airway diseases (such as chronic obstructive pulmonary disease) to cancer. Unless otherwise noted, most data discussed throughout this dissertation were collected from chronic smokers—those at greatest risk for negative consequences and who have been most studied. Smokers have greater susceptibility to and take longer to recover from viral and bacterial illness and infection (64, 153, 253), and experience impaired healing and greater risk of complications after injury and surgery (82, 117, 163). Risk for injury (212, 237, 285) and prolonged disability (182, 363) are higher in smokers. Among these risks is an increased incidence of musculoskeletal injury and delayed return to

normal activity (5, 145, 157, 182, 286, 343), though the molecular mechanisms driving these effects are currently unknown.

Smoking and General Injury Risk

Epidemiological data report approximately 2-fold greater risk for overall injury among smokers. This has been primarily described in the workplace (47-49, 212, 237, 285, 293, 339, 343, 354) and in the military (5, 145, 157, 286). Wen and colleagues (366) reported that death resulting from injury is also more common in smokers than non-smokers. This risk was dose-dependent, such that those that smoked >20 cigarettes per day had the greatest risk (>5 times greater chance of death from a work-related injury than that of non-smokers). Higher rates of injury not resulting in death, including traumatic and repetitive strain injury, have also been well documented among smokers in the workplace across cultures. For example, a greater than 2-fold risk for injury incurred at work has been reported in smokers when compared to non-smokers both in Northern France (47) and Great Britain (354).

Similar rates of injury occur across professions with varying degrees of physical demand, including those working in agriculture (212), construction (48), railway laborers (49), manufacturing (237) and postal workers (293). Heightened risk for repetitive strain injury in the upper extremities has been reported (285, 339) in smokers. Furthermore, worker's compensation claims for back injury are higher in current smokers (adjusted odds-ratio (OR): 1.64) than former (OR: 1.56) or non-smokers (OR: 1.00), suggesting a delayed recovery in smokers (363). Other

data suggest that this effect is dose-dependent, with time-loss injury being lowest in light (<11 cigarettes per day, OR: 1.6) and highest in heavy male smokers (>20 cigarettes per day, OR: 2.8)(157). Women had similar risks, with even higher risks for heavy smokers (OR: 4.4).

Smoking and Musculoskeletal Injury

While the increased risk of general injury in smokers is important to public health, the incidence of musculoskeletal injury is of greatest interest to this dissertation. Increased risk for musculoskeletal injury in smokers is particularly notable in the military (5, 145, 157, 182, 286), where recruits and active duty service-members are subjected to intense physical stresses. These effects are seen in both current and recent smokers (those who have recently quit, typically upon entering basic training), indicating that the mechanisms driving increased injury are, at least in part, persistent. During basic training, where smoking is not permitted, personnel who had smoked before entering training remained at increased risk for injury (5, 157); among those who had quit within the past year, women continued to have a higher risk (OR: 1.6), while injury risk in men decreased below that of non-smokers (OR: 0.7) (157). Altarac and colleagues (5) reported that risk varied depending on the type of injury between 1.32 (overuse) and 1.62 (traumatic) in men, with greater time lost from training after injury in smokers (OR: 1.47 for ≥ 6 d lost). Injury risk was also dose-dependent, with those who smoked the most in the past month (+1 pack) having the greatest risk (adjusted OR: 2.03) versus $\leq \frac{1}{2}$ pack having the smallest risk (adjusted OR: 1.43).

Increased risk for musculoskeletal injury has also been reported in current smokers within the military outside of basic training, when they are permitted to smoke (286). Injured active service members who smoked were more likely to be discharged due to physical disability after musculoskeletal injury (182). Rates of disability due to musculoskeletal injury are dose-dependent, ranging from 17.6% in heavy smokers down to 12.2% in non-smokers. Former smokers had a disability rate of 10.3%, slightly but non-significantly below that of non-smokers (182). However, the injuries presented in the above studies were either joint-related injuries or were not defined; therefore direct information on muscle-specific injuries remains scarce.

Effects of Smoking on Skeletal Muscle

To my knowledge, there are no published studies on the effects of smoking on direct skeletal muscle injury and recovery, although there have been a few retrospective studies reporting increased risk for complications in smokers after surgeries requiring muscle flaps (45, 80). Among the complications reported were flap necrosis and donor site complications (45). There are a handful of reports regarding the effect of smoke on skeletal muscle function (19, 162, 227, 290, 347, 370), reviewed in Degens (72). Together, these studies suggest that smokers have similar or slightly lower maximal muscle strength, may be more fatigable, and rely more heavily on anaerobic metabolism as compared to their non-smoking peers.

With regard to muscle function, mice exposed to nose-only smoke generated ~20% less force at high frequency simulations in the soleus, although this difference

did not reach significance ($p=0.08$) (290). In humans, studies have found smokers to have significantly lower strength in the quadriceps muscle group than non-smokers (19, 257). For example, in one study of middle-aged healthy non-smokers and smokers, the maximal voluntary contraction of their quadriceps was 38.50 ± 1.7 and 36.78 ± 1.5 kg, respectively. However, others have reported no difference between smokers and non-smokers for muscle strength (370).

Smoking may also impair other facets of muscle function, including fatigability. Skeletal muscle of smokers was more fatigable in response to electrical stimulation, though this was not related to level of smoking or respiratory function (370). However, mice exposed to cigarette smoke for 6mo were not more fatigable with repeated electrical stimulation, although force production at each time point was slightly lower (290). Additional data are needed to clarify whether the skeletal muscle of smokers is more fatigable than non-smokers.

Although they did not have greater fatigability, mice from this and another study did present with a shift from Type IIa to IIx/b muscle fibers in the soleus after exposure to cigarette smoke (290, 328), while several groups report smaller cross-sectional area and/or percentage of low oxidative/high glycolytic muscle fibers in smokers (162, 169, 226, 257, 294, 311). These physiological alterations to muscle suggest that skeletal muscle of smokers may not function in the same way as non-smokers, yet mechanisms underlying these phenomena remain undiscovered.

Three studies may shed some light on these findings and lend a clue to why smokers are at higher risk for injury. First, under non-exercised conditions, protein synthesis appears to be suppressed in smokers as compared to non-smokers

(fractional synthesis rate 0.037 vs 0.059, respectively). Additionally, two factors associated with muscle protein catabolism, myostatin and MAFBx, are expressed at higher levels in smokers, suggesting smoking suppresses muscle regeneration via these pathways (276). Second, a recent study reported muscle membrane damage and sarcomeric disruption at 4 and 48h after smoke exposure in rats (93). These rats experienced significantly increased expression of MyoD ($937 \pm 491\%$) and myogenin ($1029 \pm 477\%$) at 4h compared to controls, further indicating altered muscle turnover. With repeated exposure, sarcolemmal disruptions remained, with approximately 15 and 10% of fibers showing signs of disruption at 5 and 30d, respectively (compared to 28 and 16% at 0h and 4h post-exposure, respectively). Sarcomere damage increased over time from 3.8% at 0h to 4.8% at 48h and up to 25.8 and 16.4% at 5 and 30d, respectively. In the third study, mice exposed to smoke over a period of up to 32wk initially (within 8wk) experienced enhanced pro-inflammatory signaling including intracellular adhesion molecule-1 (ICAM-1) in lymphocytes and muscle C-reactive protein (162). However, more prolonged exposure resulted in suppressed expression of these genes. By 32 weeks of smoke exposure, muscle cross sectional area was significantly smaller and Type I oxidative fibers were less prevalent. Peroxisome proliferator-activated receptor (PPAR) pathway gene expression, which enhances oxidative metabolism, was suppressed. These data support a potential mechanism through which smokers may be more fatigable, and suggest that individuals exposed to cigarette smoke may experience negative effects on muscle with every cigarette inhaled. Over time, they would be less able to repair muscle, more prone to injury, and experience impaired recovery.

However, the effects of smoking on muscle damage and recovery responses have yet to be tested in a controlled laboratory experiment.

Understanding the mechanisms that increase risks for injury and impaired healing in smokers is important for improving recovery and helping to reduce injury rates and improve productivity and overall quality of life. Furthermore, knowing the underlying mechanisms that lead to these effects in smokers may help us to better understand potential modifiers of damage and repair processes in skeletal muscle.

Skeletal Muscle Damage

Skeletal muscle is a plastic tissue with the ability to repair and regenerate in response to damage. A unique aspect of skeletal muscle is that small damaging stimuli (such as those imposed by alterations in activity) typically lead to adaptation, which can result in enhanced size, specific contractile properties, overall function, and/or performance. The muscle damage response has been studied to gain insight into muscle health, function, and performance. Although much remains to be understood with regard to the muscle damage response, regeneration, and adaptation, it is clear that there is a coordinated, established, and sequential series of events drives muscle's response to damaging stimuli.

Muscle damage can occur naturally in response to a variety of stimuli, including traumatic injury (such as impact (contusion, crushing) or environmental extremes (such as freeze)), exposure to toxins, and mechanical (or strain-induced) injury. Strain injuries can range from traumatic, as in the case of a "pulled muscle,"

to low level chronic strains induced by deliberate exercise. The most common type of exercise that causes strain injuries is muscle-lengthening (eccentric) actions.

Following injurious stimuli, the response of muscle can be categorized as three principal phases: damage, repair, and regeneration. Generally, the damage/repair cycle is resolved within several weeks of the initial stimulus. Repair and regeneration can occur simultaneously, and may also be followed by remodeling of the muscle. The damage phase can be further categorized into “primary” and “secondary” damage; the former primarily results directly from the initial stimulus, while secondary damage can be an unintended consequence of inflammatory processes critical to the ultimate recovery of the muscle.

The type of stimulus that evokes muscle damage affects the overall response, including the timing and magnitude of responses, the contributions of primary versus secondary damage, the amount of damage to secondary tissues (blood supply, nerve, bone, connective tissue), inflammation, and the underlying molecular mechanisms that ultimately control the effectiveness of the repair and regeneration. To better understand the damage/repair process, several laboratory experimental models of muscle damage have been developed.

Experimental Models of Muscle Damage

Much of our understanding of muscle injury and repair comes from animal and cell culture models due to the greater ability to control independent variables. These models include: chemical exposure (for example taipoxin, cardiotoxin or notexin injection (90, 202, 292)); environmental insult (freezing (304, 324, 361,

362)), traumatic injury (crushing or penetrating injury (37, 211, 319)); and mechanical strain (stretch/strain (178, 341)). Injury can also occur during exercise and different exercise models have been used to evoke mechanical damage in both human subjects (63, 192, 222, 239) and animals (16, 121, 125), especially exercise protocols that utilize eccentric, or lengthening, muscle actions.

Eccentric exercise (ECC) elicits transient exercise-induced muscle damage (EIMD), followed by repair, regeneration, and adaptation of the muscle (reviewed in (39, 60)). The manifestations of EIMD include loss of strength, delayed onset muscle soreness, swelling, increased stiffness, and the release of muscle-specific proteins into the bloodstream. Repeated bouts of the same activity result in attenuated responses (reviewed in Howatson (129)). Popular methods for inducing EIMD include metabolically challenging models, such as downhill or downstairs running or stair stepping, and isolated eccentric exercise, for example squats or performance of maximal, eccentric, single joint movements. The last model allows researchers to isolate a specific muscle group to test the damage response and control, as much as possible, independent variables. The responses to ECC, including the manifestations and cellular and molecular signaling underlying these responses, vary depending on a number of factors, including the type of model (e.g., metabolically challenging vs isolated actions), the muscle group (e.g., elbow flexors vs knee extensors), and submaximal vs maximal actions. Generally, the most robust damage responses are those produced by isolated, maximal actions (reviewed in (60)).

There are numerous benefits of the ECC model, which can be used in a controlled, laboratory setting. The damage from ECC is transient and generally quite

safe, with subjects returning to normal activity within 1-2 weeks. As well, eccentric actions (and the resultant responses) are more common among everyday life than traumatic injury, making information gleaned through such models more applicable to the general population. Consequently, I chose to use an established ECC model to evoke damage and then study the effects of smoking on the damage response. The remainder of this chapter reviews this model in detail and postulates how smoking is could modify the response to ECC.

Eccentric Exercise Damage Response

In 1902, Theodore Hough first postulated that delayed onset muscle soreness following exercise was the product of micro-tears in muscle, elicited through mechanical stimuli such as exercise (128). Subsequent delayed onset muscle soreness (DOMS) studies found that soreness is greatest following primarily eccentric, rather than concentric or isometric actions (10, 327). In the 1980s, electron microscopy studies finally demonstrated direct evidence of physical muscle damage following ECC (100).

Current knowledge suggests that ECC places mechanical stress on the muscle, resulting in primary damage. In the days that follow, enhanced disruption to the muscle can occur, referred to as “secondary damage” and is commonly thought to result from inflammation. Inflammatory cells and molecules also stimulate signaling within the muscle to control the repair process. Remodeling and repair typically take 10-14d to complete, and healthy muscle typically adapts such that further bouts of the same exercise elicit less damage and less dramatic responses to

eccentric exercise (known as the repeated bout effect). The most commonly characterized responses to ECC are those that indirectly indicate damage: loss of strength, swelling, muscle soreness, and the leakage of muscle-specific proteins into the blood. The sections below briefly summarize each of these markers. Those markers that are significantly linked to inflammatory processes (such as swelling and DOMS) are also described in greater detail later in this chapter.

Indirect Markers of Damage

Loss of Force Production

Prolonged loss of force production (strength loss) is generally considered the best indirect measure of muscle damage, as it moderately correlates with direct measurements of damage. Force production decreases during ECC and usually resolves fully by 5-6d post-exercise, but in some cases can persist for months (299). Strength loss can be described into two phases: early loss (comprised of both metabolic fatigue from the exercise itself and potential mechanical damage to the muscle) and prolonged loss (due to structural damage and altered signaling). Strength loss can vary depending on the mode of exercise, muscle group, submaximal versus maximal actions, angle and speed of contraction, total time spent under tension, and the total volume of exercise, as well as between individual subjects.

The relative level of post-exercise strength loss tends to be greatest with maximal, forced eccentric actions of the elbow flexors, and can reach up to 90% decrease from baseline with higher numbers of actions (59). The time to full

recovery is generally linked to the amount of damage and magnitude of initial force loss. The ability to maintain force also drops off rapidly with muscle damage; thus, the initial force output can be sustained for only a short period of time, after which force decreases more quickly than in undamaged muscle (reviewed by Warren (359) and Hyldahl and Hubal (135)).

Swelling

Swelling of the exercised limb typically peaks 3-6d after ECC (63, 123, 222). As edema resolves, the swelling moves distally along the limb as the additional fluid is reabsorbed. Swelling may not be noticeable, depending on the affected muscle compartment. For example, the elbow flexors are more prone to obvious signs of swelling than muscles of the anterior upper leg—this may be due in part to the expandability of the muscle compartment as well as the amount of damage to the muscle. There is also high variability in the extent of swelling. Because swelling does not correlate well with any other response to ECC, the reason for this variability is currently unknown. Swelling is believed to be the result of inflammatory processes that take part in the regeneration of a fully functioning muscle after ECC, which will be summarized in more detail later in this chapter.

Delayed Onset Muscle Soreness

Delayed onset muscle soreness (DOMS) is a phenomenon specific to muscle damage and describes hyperalgesia (increased sensitivity to pain) that is delayed in its onset (occurring some hours after exercise) and typically resolves within less

than a week of the initial damaging event. Reported soreness can be severe and may interfere with activities of daily living. The overall magnitude and duration of DOMS does not correlate well to the level of muscle damage incurred (via direct measure); however, the level of peak soreness does grossly relate to the level of damage, with higher peak soreness occurring with greater muscle damage.

Current theory suggests the hyperalgesia underlying DOMS is mainly caused by inflammatory mediators and related signaling proteins (reviewed in Hyldahl and Hubal (135)). Thus far, attempts to suppress DOMS have been largely unsuccessful, with the primary exception being the use of non-steroidal anti-inflammatory drugs (NSAIDs). However, NSAID use during muscle recovery can attenuate functional regain and suppress adaptation (168, 223). The failure to adapt with use of NSAIDs illustrates how enmeshed the underlying mechanisms of response to ECC are—by suppressing inflammation and therefore DOMS, muscle recovery and adaptation can be inhibited. For additional information, the reader is referred to reviews by Baldwin Lanier (14); Schoenfeld (303); and Stone and colleagues (321). Specific mechanisms of inflammation that drive DOMS are explored in greater detail later in this chapter.

Elevated Blood Levels of Muscle-Specific Proteins

The leakage of muscle-specific proteins into the bloodstream through the damaged membrane is another indirect indicator of muscle damage. The most commonly measured proteins in blood after ECC are creatine kinase (CK) and myoglobin.

CK is a two-subunit enzyme critical for energy (ATP) production. Originally, the CK-MB variant was used to confirm myocardial infarction. In the middle of the 20th century, the association of skeletal muscle damage with increased CK (MM variant) was noted, and in the 1960s altered blood CK activity was associated with exercise. After ECC, serum CK activity peaks approximately 5d post-exercise, though the level of release varies widely among specific models of eccentric exercise and among individuals (61). While a good indicator that damage has occurred, CK activity in the blood does not correlate well with the level of muscle damage itself (203), nor with other indirect markers of muscle damage like strength loss.

Myoglobin transports oxygen and carbon dioxide within muscle tissue. After ECC, myoglobin leaks into the bloodstream, where it then travels to the kidneys for excretion. Unlike high CK levels, which are considered relatively harmless, high levels of myoglobin outside of skeletal muscle can be dangerous, if not deadly—if not cleared effectively, myoglobin can precipitate in the kidneys, leading to renal failure, a phenomenon called exertional rhabdomyolysis. Myoglobin peaks in the bloodstream after ECC at approximately the same time as CK, although it can peak slightly later. Myoglobin release is also much lower in magnitude than CK, though the accumulation of the two proteins tends to correlate well. Because myoglobin is expensive to measure and post-ECC CK and myoglobin levels correlate well, CK is often used as a surrogate for myoglobin.

Direct Markers of Damage

In 1988, Friden (100) first reported histochemical evidence that ECC resulted in muscle damage. In the following years, a number of other researchers reported similar findings with ultrastructural damage and loss of sarcomere organization (z-line streaming) (98, 146, 240). Disturbances to both the sarcolemma and extracellular matrix (muscle connective tissue) have been reported (159, 181). It was hypothesized in 1990 that, during lengthening actions, damage is caused when sarcomeres are stretched beyond the point where actin and myosin cross-bridges can hold. Known as the “popping” sarcomere hypothesis (228, 230), this theory does not explain the additional, or secondary, disruption to muscle structure seen in the days following exercise.

In the early 2000s, Yu and colleagues noted significant structural disruption to a number of structural proteins, including desmin (380, 381, 383), following ECC. These alterations persisted and even worsened over the course of approximately one week after exercise before resolving. These disruptions occurred in areas with sarcomere insertions, indicating sarcomerogenesis and restructuring of proteins, rather than damage, in the muscle. Another large difference between ECC and other models of muscle damage is the lack of associated cell death. In humans under voluntary exercise conditions, there is little evidence to suggest that ECC results in disruption to the sarcolemma to the extent that would result in the overt death of the muscle cell (reviewed in Malm (197)).

Adaptation

Healthy muscle adapts to repeated exposure to ECC. Remodeling of the muscle has been reported at the ultrastructural level, with sarcomere insertions (229) and alterations to intermediate filaments (97). Further, there is remodeling of the connective tissue (167) and the extracellular matrix, the network of proteins that connect the contractile elements to the surrounding connective tissue (189). Over time, these changes in protein likely lead to larger adaptations (including the insertion of sarcomeres) and can drive muscle hypertrophy. Other adaptations may occur depending on the specific nature of the exercise utilized, such as shifts in metabolic preference. These alterations to ultrastructure and connective tissue provide evidence of not only repair, but also remodeling and adaptation to the structure and function of the muscle.

Repeated bouts of ECC within several months yield a reduction of gross responses (62, 246, 249, 323). This protective “repeated bout effect” (RBE) was first characterized in the 1980s and it is now widely recognized that repeated performance of the same ECC activity results in attenuated DOMS, muscle protein release, and strength loss (41, 127, 129, 246). More recently, the cellular and molecular changes that may influence RBE have been investigated, including inflammatory signaling (133, 323) and the effect of ECC on the contralateral limb (130, 372), yet the underlying mechanisms remain poorly understood. Still, long-term gross adaptations to consistent application of eccentric actions, such as hypertrophy (122, 170) and increased strength (122, 147, 170), are well documented. For additional information, the reader is referred to reviews by Proske

and Morgan (283) and Vogt and Heppeler (352) on muscle adaptation and McHugh (213) regarding RBE. Specific roles for inflammation in skeletal muscle adaptation will be discussed later in this chapter.

Mechanisms Driving Damage and Repair

The following section provides a framework to understand the processes driving the EIMD response (55, 67, 116, 133, 137, 156, 192, 194). An early review by Armstrong (7) proposed a four-stage response to muscle injury: initial, autogenic, phagocytic, and regenerative. Since that time, our understanding of these processes has evolved to the following three-stage theoretical framework of the EIMD response: 1) the initial stimulus stresses the muscle and results in “primary” (mechanical) muscle damage; 2) a “secondary” damage stage, driven by alterations to the inflammatory balance, that eventually resolves with repair and regeneration; and 3) remodeling to the muscle ultrastructure that occurs along with and following secondary damage, and is an important component to regeneration and adaptation to future perturbations.

Primary EIMD is largely the result of mechanical mechanisms. The experimental model used to evoke damage will greatly vary the extent and characteristics of the damage response including the specific underlying signaling pathways, though the major events that follow generally remain consistent among models of muscle damage. These events have some common elements with damage induced via non-exercise means (crush, freeze, or toxin-induced injury), though other aspects of the response are unique to the damage-inducing stimulus.

Secondary damage can be generally split into two phases: the “early” (mostly pro-inflammatory) phase, characterized by increased damage to muscle; and the “late” (mainly anti-inflammatory) phase, typically associated with the stimulation of tissue repair. A critical component of the damage/repair cycle is a shift in the inflammatory balance—from early pro-inflammatory processes towards favoring anti-inflammatory processes for subsequent repair and regeneration. Without this natural progression, repair and regeneration would be more prolonged and adaptation likely attenuated, if not completely abolished. Therefore, understanding the processes that drive secondary damage, regeneration, and remodeling, such as inflammation, is critical to promoting effective muscle healing and function, and is a major focus of this dissertation and the remainder of this chapter.

Inflammation and Muscle Damage

In 1962, Brendstrup (31) suggested that inflammation occurred after ECC as a result of muscle damage, specifically noting the presence of edema in rabbit triceps surae post-exercise. The infiltration of inflammatory (specifically mononuclear) cells into damaged muscle was first noted approximately 20 years later (8, 146). Since that time, our knowledge of inflammation in muscle damage and regeneration has grown substantially. However, the diverse type and nature of muscle damage models used in research (for example, downhill running, submaximal vs maximal isolated actions, voluntary vs electrically stimulated) result in high variability within the overall inflammatory response, making understanding the role of inflammation in EIMD more challenging. What is clear is that a sequential

cascade of inflammatory events occurs in response to a damaging stimulus and that this inflammatory process can cause secondary damage to tissue.

The conspicuous manifestations of inflammation after ECC are swelling of the injured muscle or limb and hyperalgesia (increased sensitivity to pain). The hyperalgesia is delayed, and is associated with the sensation of prolonged soreness that peaks 24-48h post-ECC (DOMS). Swelling and DOMS are believed to be a result of inflammation, although they do not relate to one another in magnitude or temporally. As mentioned previously, swelling typically peaks 3-6d after ECC while DOMS generally peaks at 24-48h post-exercise.

At the cellular level, inflammation is characterized by the release of pro- and anti-inflammatory cytokines and chemokines (for example tumor necrosis factor (TNF)- α and interleukin (IL)-10) into circulation. These chemical messengers stimulate the migration of leukocyte populations to the site of injury, their extravasation into the muscle, and their activity. Neutrophils and macrophages are the primary leukocytes involved in EIMD. Neutrophils are primarily pro-inflammatory, and neutrophil buildup can enhance damage to the area. Macrophages can be predominantly either pro- or anti-inflammatory, playing diverse roles in the balance between damage and regeneration. Macrophages in the early stages after ECC are predominantly pro-inflammatory (known as ED1 or M1), while at later stages they tend to be anti-inflammatory (ED2 or M2).

The underlying molecular signals that control inflammation, secondary damage, and regeneration across time after ECC are complex and poorly understood. A number of important signaling pathways have been reported to play

important roles, such as the nuclear factor kappa beta (NF κ B), and the mitogen activated protein kinases (MAPKs) pathways including the extracellular regulated kinases (ERKs), as well as related cytokines and extracellular signaling proteins such as the interleukins and nerve growth factor (NGF). These pathways and proteins will be explored in more detail later in this chapter.

The following section summarizes a typical post-ECC inflammatory profile, including how inflammation is related to damage markers (soreness, swelling), cellular level changes (cytokine and chemokine release, leukocyte activity and numbers) and changes taking place at the molecular level (gene and protein expression in muscle).

Inflammation and Swelling

Swelling after ECC can be considerable and is sometimes recognizable to the naked eye. Initially, and most commonly, this inflammatory response was measured by recording changes in limb circumference, although attempts to determine the specific location and source of the increased limb circumference has led to the use of muscle biopsy to test for swelling of individual muscle fibers (99, 382), slit catheter technique (99), and the non-invasive practices of magnetic resonance to test cross-sectional area as well as tissue density with transverse relaxation (T2) and ultrasound (57, 247). Research suggests that swelling can result from: 1) the increase in fluid around the site of injury, either within the muscle or subcutaneous tissues (57, 94, 247); and/or 2) swelling within individual muscle fibers (99, 382).

Swelling is most commonly reported: 1) with a greater extent of damage; 2) with models that utilize maximal exertion and are less metabolically challenging; and 3) in the elbow flexors (which are within an elastic and expandable compartment as compared to muscles in tighter compartments such as the plantar flexors). Extreme cases of swelling after ECC have been reported that were not related, either temporally or by magnitude, to other indicators of damage such as strength loss, DOMS, or CK release. However, in some of these cases, the subjects were high responders for most, if not all, muscle damage indicator parameters (300). As previously mentioned, swelling generally appears to be unrelated to other indicators of muscle damage, and this makes it difficult to determine the underlying cause for such changes.

Swelling has also been studied using imaging techniques. Changes to tissue density immediately after ECC have been reported in MR studies examining alterations in T2 relaxation time, essentially a measure of fluid within tissue or tissue density. Early alterations in tissue density are followed by a second, delayed T2 peak that typically arises 12-24h after ECC and generally peaks 3-6d post-ECC (57, 61, 94, 247, 326). Overt swelling typically resolves by moving down the limb in the direction of gravity and fluid is eventually resorbed by the lymphatic system (247). However, changes in T2 can persist up to 23d post-ECC, when all other signs of swelling have dissipated, suggesting that changes to tissue density after ECC may be a sign of remodeling (247), or that T2 is a more sensitive measure than others to detect persistent alterations of fluid within muscle. While these studies have indicated the physical source of changes in limb circumferences (fluid increase

within the muscle, within the fibers themselves, and in subcutaneous tissues), the mechanisms that result in swelling have yet to be specifically identified. Furthermore, swelling is not strongly related to hyperalgesia and DOMS (which tend to resolve before significant swelling occurs).

Inflammation and Delayed Onset Muscle Soreness

One of the hallmark effects of ECC that is related to inflammation is delayed onset muscle soreness (DOMS), delayed hyperalgesia that typically appears within 12h, peaks 24-48h, and resolves by 4 to 7d. DOMS is often the most noticeable and bothersome “symptom” of muscle damage. Previous exposure to the same eccentrically-biased activity within ~6mo (RBE) attenuates the magnitude of DOMS, making earlier exposure to ECC the most effective protection against future DOMS.

The magnitude and timing of DOMS reported varies widely among individuals and can depend on the level of damage, the model of ECC, and the muscle group exercised. Magnitude and duration are typically (but not always) linked, such that greater peak soreness tends to be more prolonged. Generally, greater muscle damage results in greater soreness, although the level of soreness does not always reflect the magnitude of damage (251). DOMS is also affected by the model of ECC—greater perturbations yield more soreness. For example, downhill running typically elicits lower soreness than concentrated eccentric actions of the knee extensors, and maximal contractions lead to greater soreness than submaximal ones.

As previously discussed, interruption of DOMS through the use of NSAIDs can interfere with muscle healing time and adaptation (221, 223). This indicates the

importance of the complex and coordinated inflammatory response post-exercise to muscle regeneration. Post-ECC cellular and molecular alterations have been identified (55, 67, 116, 133, 137, 156, 192, 194), including those involving inflammation and secondary damage that have furthered our knowledge of muscle damage and repair considerably. The current understanding regarding the mechanisms that mediate DOMS are briefly summarized below. For a more detailed review, the reader may also see Hyldahl and Hubal (135). The following sections summarize inflammatory changes following ECC that take place at the cellular level.

Cellular Inflammation Associated with Muscle Damage

Leukocyte infiltration into injured muscle generally peaks within the first 3-24h post-exercise for neutrophils (216) and 24-48h post-exercise for macrophages (21, 88, 120, 267). These cells serve to remove debris (50, 79) and can signal molecules to promote remodeling and regeneration (325). Macrophages are classified as either ED1/M1 (primarily pro-inflammatory action) or ED2/M2 (primarily anti-inflammatory action) macrophages. Earlier research classified the polarization of macrophages into ED1 or ED2 by the presence or absence of two antigen markers on the cell surface: CD68⁺/CD163⁻ (ED1) or CD68⁻/CD163⁺ (ED2). More recent research has revealed greater specificity and complexity in phenotypic characteristics of skeletal muscle macrophages after ECC; therefore, macrophages involved in skeletal muscle damage are now most commonly classified as M1 or M2.

M1 macrophages peak approximately 24h post-ECC and are involved in phagocytosis and cytokine signaling as part of the early phase of inflammation. The

the late phase of inflammation emerges at approximately 2d post-ECC, with a shift from pro- to anti-inflammatory cytokine signaling (for example, a decrease in TNF- α and increase of IL-10) that is brought about by the appearance of a second wave of M2 macrophages. Macrophage polarization shifting from M1 to M2 likely plays a role in the shift from potentially causing secondary damage to rebuilding because M2 macrophages outnumber M1 macrophages around the same time as gene expression of satellite cells shifts from proliferation to differentiation. Furthermore, M2 macrophages produce and release growth factors that play a significant role in regeneration, such as TGF- β (86). For extensive reviews of cellular inflammatory changes following muscle damage, the reader is referred to reviews by Peake and colleagues (270); Pilon and colleagues (280); Butterfield and colleagues (40); and Chargé and Rudnicki (46).

Early Phase Inflammation

The early phase of inflammation in the ECC model begins during exercise and typically lasts up to approximately 2d post-ECC. Under normal conditions, skeletal muscle contains resident macrophages that are quiescent within the epimysium and perimysium. As previously mentioned, macrophages are heterogeneous, particularly among tissue-specific resident macrophages. The classification of skeletal muscle resident macrophages in humans remains poorly defined, save that they differ from invading inflammatory (M1/M2) macrophages in their antigen presentation.

After ECC, resident macrophages are activated and, along with muscle fibers and other non-muscle cells, release chemical messengers (such as monocyte

chemoattractant protein (MCP)-1 (133, 166, 255)) to evoke the activation, movement, and extravasation of inflammatory cells from circulation into the site of injury. The first cellular responders (peaking at 6h post-ECC) are the neutrophils, polymorphic leukocytes that serve to identify and break down damaged tissue through phagocytosis and respiratory bursts, resulting in damage and death to foreign pathogens, damaged tissue, and healthy neighboring tissue. Neutrophils are pro-inflammatory and their accumulation post-ECC can result in secondary tissue damage. After neutrophils accumulate, monocytes begin to infiltrate and make the phenotypic transformation into macrophages. During the early inflammatory phase, M1 macrophages propagate pro-inflammatory processes.

Early Phase: Cytokine and Chemokine Signaling

The physical movement of circulating leukocytes is initiated by stretch-activated calcium channels (69) that result in the release of cytokines and chemokines into circulation. These molecules initiate the proliferation and chemotaxis (movement) of leukocytes to the site of injury. The first cytokines to be released during the first ~48h after maximal ECC in humans are the pro-inflammatory cytokines TNF- α , MCP (325, 358), and IL-1 (188, 248), followed by IL-6 (34, 112, 313), and the colony stimulating factors (CSFs) (123, 266, 313) from resident macrophages, skeletal muscle, and infiltrating leukocytes. Together, these cytokines promote activation, proliferation, and chemotaxis by altering gene expression and presentation of adhesion molecules of leukocytes, stimulating them to roll along the surface of the vasculature and extravasate into the muscle. It is

important to note that not all studies have shown an increase in TNF- α (123, 313), although this may be a function of the location and timing of measurement and differences in exercise mode.

Systemic alterations in cytokine levels may be more difficult to detect due to dilution in circulation. Furthermore, research has consistently shown that the extent to which cytokines are expressed varies according to the type and intensity of exercise performed, as well as temporally. Because of this variability, it is not clear whether altered levels of circulating cytokines are sufficient or necessary to evoke major responses after ECC, though changes to cytokines near the site of injury can still affect regeneration. TNF- α functions by chemoattracting satellite cells to damaged regions of muscle in mice (340). IL-6 stimulates proliferation of satellite cells after ECC (356), though this cytokine also plays both anti- and pro-inflammatory roles under other conditions.

Early Phase: Neutrophil Migration and Infiltration

Neutrophils are the first leukocytes to arrive at the damaged muscle (187, 188, 210), infiltrating within an hour after ECC. After ECC, there is a transient increase in neutrophil counts, which peaks in circulation at approximately 3-6h post-exercise (89, 198, 266, 281, 298). Enhanced levels of cytokines and chemokines in plasma, such as TNF- α (273, 337) and G-CSF (266), result in the chemotaxis and diapedesis of neutrophils into the muscle. Physical alterations assist diapedesis, including the deformation and reorganization of neutrophils (376, 377) and the endothelium (264) through modifying adherent junctions between cells (73).

Muscular accumulation of neutrophils peaks between 6-24h after ECC, as measured by biopsy (21, 88, 207, 284, 323) and the use of radio-labeling (186). Following entry into the muscle, neutrophils contribute to pro-inflammatory processes through the release of cytokines and chemokines, as well as signaling stimulated by respiratory, or oxidative, bursts. Accumulation of neutrophils can also stimulate secondary tissue damage.

Neutrophils mediate two important processes: degranulation and phagocytosis. In direct phagocytosis, debris is engulfed by neutrophils and sequestered in phagolysosomes, where noxious substances destroy the debris. Any leftover material is then released from the neutrophil for further degradation or recycling via degranulation. Neutrophils also release noxious substances into the extracellular space through several types of degranulation. These noxious substances, released as part of respiratory bursts, can result in damage to surrounding healthy tissue (secondary damage) and include nitric oxide (241), although others are involved. For example, myeloperoxidase (MPO) release from neutrophils modulates membrane disruption by way of nitric oxide and its derivatives.

Generally, neutrophils break down damaged tissue and remove debris. However, the release of TNF- α from neutrophils assists in the chemoattraction of satellite cells and M1 macrophages (340), both of which are important in the regeneration process but serve opposing purposes of regeneration and (primarily) additional damage, respectively. Oxidative molecules signal cascades related to muscle repair (148) and growth such as insulin-like growth factor (IGF)-1,

phosphoinositide 3-kinase/protein kinase B (PI3-K/AKT), NFκB, and the MAPKs (114, 141, 302), and may therefore play an important role in subsequent regeneration. Therefore, neutrophils may also indirectly assist in muscle regeneration through the release of radical species. Neutrophils ultimately initiate apoptosis and die between 1-2d after ECC. These apoptotic cells are then phagocytosed by infiltrating macrophages (289).

Early Phase: Macrophage Activation, Mobilization and Infiltration

After an early period of neutrophil invasion, the extravasation of monocytes begins (267). Following their entry into the muscle, they transform into inflammatory macrophages. Monocyte classifications are reviewed in Yona (379). In mice, monocytes are classified by the expression of the cell surface marker Ly-6C; monocytes that are Ly-6C^(high) are classified as pro-inflammatory while those that are Ly-6C^(low) are classified as anti-inflammatory. In humans, the expression of the cell markers CD14 and CD16 generally determines the classification of monocytes: pro-inflammatory express CD14^(high)/CD16^(neg), while CD14^(low)/CD16^(high) are considered anti-inflammatory. However, monocytes and macrophages are a highly heterogeneous population in terms of their actions and antigen expression, both of which can vary substantially depending upon the tissue type in which they reside, and therefore caution must be taken to classify these cells. In humans, the classification of monocytes based on their expression of CD14 and CD16 is not well characterized and therefore I discuss them here by Ly-6C expression, only. In response to injury, Ly-6C^(high) monocytes extravasate first and are most likely the

pro-inflammatory precursors to the phenotypically pro-inflammatory macrophage population (M1) (154). Once they have infiltrated the muscle, these monocytes transform into macrophages through the expression of specific antigens, including CD68 and CD86 (M1), and CD206 and CD163 (M2); most macrophages that enter muscle during the early inflammatory phase become M1 phenotype (reviewed in Tidball (337)).

The majority of macrophages in this early phase (M1) phagocytose debris, although they are also involved in cytokine signaling through the release of IL-6 and TNF- α . In addition to their roles as phagocytic cells, M1 macrophages have been shown *in vitro* to promote the proliferation and suppress the differentiation of muscle progenitor cells (9). Thus, M1 macrophages may assist muscle regeneration.

At early stages of healing in other tissues, macrophages may play other roles in addition to or instead of phagocytosis and cytokine signaling—for example, they have been shown to release TGF- β and vascular endothelial growth factor (VEGF)- α during wound healing, proteins that stimulate muscle cell differentiation and angiogenesis, respectively (185). However, the timing of events may be different between healing in other tissues and muscle regeneration. For instance, when macrophages are co-cultured with necrotic muscle progenitor cells *in vitro*, the phagocytosis of muscle cell debris leads to macrophage production of TGF- β and a subsequent phenotypic shift to M2 macrophages. Generally, M2 macrophages express TGF- β to a greater extent than M1 cells, and, *in vivo*, M2 macrophages are most prevalent during the later stage of inflammation (9), resulting in an increase of TGF- β and enhanced muscle regeneration.

M1 macrophages can undergo a phenotypic change in response to their phagocytic activity—particularly phagocytosis of apoptotic neutrophils or debris caused by neutrophilic degranulation (9). These cells are considered to be primarily anti-inflammatory in nature and begin to express cytokine profiles more similar to M2 macrophages, including increased expression of TGF- β and IL-10 and decreased expression of TNF- α and IL-1 β (9). The phenotypic shift from M1 to M2 is a part of an important turning point in the post-injury response—a tipping of the inflammatory balance from primarily pro-inflammatory to anti-inflammatory, as well as a shift in muscle progenitor cell proliferation to differentiation, and ultimately an attenuation of secondary damage and promotion of tissue regeneration. Shifts to this final stage begin at approximately 2d after injury or stress to the muscle and are orchestrated in part by the M2 macrophages.

The Shift from Early to Late Phase of Inflammation following Damage

At approximately 2d post-ECC, the shift from pro- to anti-inflammatory cytokines coincides with the increases in the expression of IL-1 receptor- α (R- α), IL-4, and IL-10 (272, 313), which collectively block the production of IL-1, -6, and -8. Corresponding temporally with these cytokine changes is a shift in macrophage phenotype (337). Toward the end of the early phase, the neutrophils have either undergone apoptosis or degranulation and the debris is either shunted out of the cell in lysosomes or engulfed by the M1 macrophages. Research suggests that the phagocytosis of neutrophilic debris by M1 macrophages stimulates a shift in the macrophage polarization, resulting in their phenotypic transformation to M2

macrophages (289). A heterogeneous population of both macrophage phenotypes exists at this transitional phase. As the transition period progresses, the balance tips such that the M2 macrophages predominate over M1 cells. M2 macrophages release additional anti-inflammatory cytokines, further enhancing anti-inflammatory activity, quenching pro-inflammatory actions, and limiting secondary damage.

Late Phase Inflammation

The late phase of inflammation is less well understood than the early phase and is a major focus of this dissertation. Fewer studies have been performed beyond the first 24h post-ECC, and the high variability among responses to ECC is most exaggerated in the late phase, due, in part, to the wide variety of models used to elicit muscle damage and variability in response timing, and partly to the phenotypic heterogeneity of the macrophage population at this time point. The overall inflammatory events taking place two or more days after ECC have been identified; yet the specific underlying mechanisms can vary, and ultimately may significantly alter the course of recovery and regeneration.

Late Phase: Cytokines and Chemokines

As previously discussed, at approximately 2d after ECC there is a shift in the inflammatory balance to attenuate pro-inflammatory action and promote regeneration. This occurs with the combined release of IL-1R α , IL-4, and IL-10 to block the production of pro-inflammatory cytokines including IL-1 and IL-6 (337). IL-13, which plays a role in macrophage phenotype determination, may also be

released at this time. Both TGF- β and IL-10 are released at this time, which serve to enhance myogenesis. In addition, TGF- β may promote angiogenesis and rebuilding of the extracellular matrix (ECM) (84, 295). The role of these cytokines appears critical to effective healing, such that dysregulation of TGF- β or IL-1 β may promote fibrosis within skeletal muscle, leading to decreased function and increased risk of future injury (179, 204).

Late Phase: M2 Macrophages, Inflammatory Resolution, and Regeneration

During late phase inflammation, the primary macrophage population is M2, which most likely originate from the extant M1 population via polarization change or from newly infiltrating monocytes (most likely CD14^(low)/CD16^(high)). Resident macrophages could conceivably proliferate and contribute to the M2 population, although this possibility has yet to gain substantive support.

There are three subtypes of M2 macrophages, each with different functions. These subtypes are the result of the specific cytokine environment at the time of differentiation and promote distinct healing outcomes. For example, IL-13 is thought to result in macrophage phenotypes that suppress inflammation, while those activated by IL-4 and IL-10 are thought to promote tissue repair (107). M2a macrophages result in fibrotic deposition, play a role in the rebuilding of ECM and other connective tissues, and appear during the most advanced stages of healing (351, 371). This population of macrophages is thought to result from activation via IL-4 or -13 signaling, which in turn results in the activation of STAT6 (113, 183). M2b macrophages have immune regulatory functions and are less likely to play a

significant role in skeletal muscle repair, although they can be anti-inflammatory through their expression of IL-10 (29). Finally, M2c macrophages result from stimulation by IL-10 or glucocorticoids. They appear relatively early within the late phase, and are primarily anti-inflammatory through the release of IL-10 and TGF- β (9). These findings indicate the importance of quenching pro-inflammatory pathways to resolve inflammation and promote healing.

Macrophages are critical to regeneration, as the ablation of monocytes impairs regeneration (9). In addition, there must be a resolution of inflammation. Without the deactivation of pro-inflammatory activity, tissue remodeling is attenuated and this can result in fibrosis rather than myogenesis (204). In addition to previously described roles, inflammatory macrophages participate in muscle regeneration through enhancing proliferation of myogenic cells, shown *in vitro* (9, 50, 315) and, *in vivo* (22). Tidball and Wehling-Henricks (338) reported *in vivo* macrophage depletion at 2d after muscle re-loading resulted in impaired muscle regeneration and suppressed expression of myogenic genes in muscle progenitor cells (MPCs). It is thought that M1 macrophages stimulate MPC proliferation while M2 macrophages support their differentiation (9, 275). Furthermore, the signaling molecules released by macrophages and neutrophils, such as TNF- α , help drive MPC activation.

The Role of Muscle Progenitor Cells in Inflammation

Upon activation, muscle progenitor cells (MPCs; primarily satellite cells) proliferate, migrate toward the site of injury or stress, and can directly fuse with the

damaged myofiber and release growth factors and other signaling molecules to enhance regeneration and remodeling. Generally, satellite cell numbers increase by 24h post-ECC and remain elevated up to or beyond 8d, indicating a possible role in the rebuilding process (66, 67, 78, 221, 256, 268) (214, 215). These cells can also be induced without obvious signs of muscle damage or growth (66, 67, 190, 221).

Several inflammatory mediators alter the behavior of satellite cells. IL-6 deficiency in mice significantly attenuated satellite cell proliferation following muscle damage, which was rescued via paracrine IL-6 production by myotubes, *in vitro* (308). These data suggest that IL-6 signaling is critical to satellite cell activity in muscle repair. Exogenous TNF- α application *in vitro* and injection *in vivo* enhances satellite cell activation and proliferation, suggesting that it plays a role in satellite cell proliferation (180).

A number of inflammatory pathways that affect satellite cell activity after ECC also sensitize nerve endings and are therefore implicated in hyperalgesia. The most studied of these pathways is the cyclooxygenase (COX) pathway (25, 26, 219), which stimulates prostaglandin synthesis. However, it is unclear if COX is necessarily related to muscle regeneration, as there is evidence that both supports (35, 36, 365) and refutes (38, 221) a role for the COX pathway in regeneration after ECC. Another nerve-sensitizing molecule implicated in MPC activity is nerve growth factor (NGF). *In vitro* treatment with NGF suppresses differentiation of MPCs, yet improves the efficiency of MPC engraftment when these cells are transplanted into mouse dystrophic muscle (171). Other research suggests that NGF may promote

MPC fusion through the NGF low-affinity receptor, p75^{NTR}, the expression of which is increased throughout regeneration.

The sections above explored the responses of muscle to ECC at the gross physiological and cellular levels. To further delineate what specific factors drive damage and repair, we must tease apart molecular mechanisms that underlie changes at the more macroscopic level. After ECC, alterations to mRNA and protein expression occur, not only within skeletal muscle but also a variety of cell types such as MPCs, fibroblasts, and inflammatory cells.

Molecular Mediators of Muscle Damage and Inflammation

A number of studies investigating skeletal muscle damage mechanisms have resulted in the generation of large gene and protein expression profiles through the use of high throughput technologies such as microarrays (transcriptomic) (55, 137, 161, 192, 195) and 2-D DIGE (proteomic) (200)(reviewed in Gelfi (105)). Key pathways that are differentially regulated early after ECC include those involved in the stress response (195, 346), such as the heat-shock proteins (161), and the ubiquitin-proteasome pathway (161, 195, 346). At 24h the expression of structural proteins is also upregulated (161), suggesting that it may be a part of the remodeling process. The expression of inflammatory genes is altered early after ECC (55, 195), although only a small number of studies have investigated the early to late shift and later stages of inflammation (191, 192, 335).

The use of varied damage models is a major challenge to understanding the underlying molecular mechanisms that mediate responses to ECC, including

inflammation, secondary damage, and repair. For example, downhill running stimulates a different inflammatory profile as compared to isolated maximal eccentric actions, due to differing mechanical and metabolic demands. Therefore, caution must be taken in generalizing results based on the data from the small number of studies performed at later time points.

Nevertheless, a set of genes and pathways important to muscle remodeling have been identified, including those involved in muscle formation (such as the myogenic regulatory factors (MRFs) Myf5, MyoD, myogenin, and MRF4), modulation of transcription that play roles in damage and repair, and inflammation (including cytokines and chemokines). Due to the complex nature of signaling and the variety in models, it is difficult to clarify how inflammation exerts its actions upon repair after ECC. However, several inflammatory mediators and pathways have been identified as possible major players in muscle regeneration, including several of the interleukins, TGF- β , the COX-2 pathway, ERK, and NF κ B.

Inflammatory Pathways Mediating EIMD

The production of inflammation-related mRNAs and proteins can change without typical signs of inflammation or damage, including swelling, circulating cytokines and chemokines, or the infiltration of leukocytes at the site of injury or muscle stress. The specific roles inflammation may play in ECC recovery are not well understood, though several have been identified. For example, the expression of cytokines, including IL-8 (35), IL-1 β (43, 112), TGF- β (112), is altered following ECC, the production and release of which have been previously discussed. IL-6 and its

receptors (mRNA and protein) tend to increase after ECC, and may play a dual role as both pro- and anti-inflammatory mediators (35, 112, 214). This inflammatory molecule is released not only by skeletal muscle and satellite cells, but also fibroblasts (43)—cells that also release IL-1 and IL-8, suggesting a potential role in promoting inflammation (43).

Inflammatory signaling, including but not limited to IL-6, TNF- α , COX-2, ERK, and NF κ B, is involved in promoting cell proliferation, an important aspect of regeneration. Differentiation may be facilitated by both the suppression of proliferative pathways (such as ERK and NF κ B), as well as the activation of other pathways including TGF- β and VEGF- α . Other signaling pathways, such as those mediated by NGF, may promote proliferation or differentiation through the activity of downstream targets dependent on factors including which receptor the protein binds. Transcription factors, such as ERK and NF κ B, may also promote the transcription of structural proteins for remodeling. While many pathways exert inflammatory signaling that mediates EIMD, below I focus on the effects of ERK and NF κ B.

Nuclear Factor Kappa Beta Signaling Related to Inflammation and EIMD

NF κ B pathway activity increases in response to ECC and plays a role in muscle regeneration. NF κ B is a family of transcription factors that, upon activation, translocate to the nucleus and drive changes in gene expression for a variety of physiological processes, including proliferation, differentiation and apoptosis. There are two primary pathways through which NF κ B acts: the canonical (classical)

pathway, which is characterized by the v-rel avian reticuloendotheliosis viral oncogene homolog A (Rel-A)/p65 subunit binding to either p52 or p50, and the non-canonical pathway in which Rel-B binds with p52. For the purposes of this literature review, I will refer to these pathways as p65 (canonical) and Rel-B (non-canonical). The two pathways act upon different downstream targets and it is hypothesized that both pathways play a role in muscle remodeling after damage.

The activity of p65 is increased shortly after muscle damage (136) and then declines over time (13). The p65 complex inhibits muscle cell differentiation (13), can suppress myogenesis by inhibiting MyoD expression (110) and promoting the expression of Cyclin D1 (109), and may play a role in enhancing proliferation (132) (reviewed in Dolcet (76)). The role of Rel-B in muscle regeneration is less well characterized than that of p65. Rel-B activity increases as p65 activity decreases (13). Current research does not yet suggest a direct role of Rel-B in muscle regeneration, though p65 may promote proliferation of MPCs by interacting with other pathways that act in myogenesis, such as PI3K/AKT and ERK pathways (33, 83, 205).

The canonical NF κ B pathway is an important inflammatory regulator and is considered by many to be a keystone for pro-inflammatory processes. This pathway is associated with a number of chronic inflammatory conditions including inflammatory bowel disease and rheumatoid arthritis (reviewed in (18)). p65 is activated by pro-inflammatory cytokines, including TNF- α and IL-1, leading to the production of a number of inflammatory molecules after injury or muscle stress, including the cytokines and chemokines IL-1, IL-6, IL-8, MCP-1, and G-CSF (24, 175).

Through this signaling, NF κ B acts in a positive feedback loop to promote additional pro-inflammatory signaling and NF κ B activation. It has also been implicated in macrophage activity, polarization, and the transformation from monocytes to macrophages.

Rel-B activity has been associated with the resolution of pro-inflammatory processes. Specifically, inhibition of Rel-B results in continued activity of the p65 NF κ B pathway as well as the continued production of pro-inflammatory cytokines (11, 334). Furthermore, overexpression of Rel-B suppresses pro-inflammatory mediators including IL-6 and COX-2 (218). These data, along with knowledge that activation of the canonical and non-canonical pathways shift along with the inflammatory balance after ECC, strongly support the role of the NF κ B pathways in the development and resolution of inflammation after ECC. These pathways also interact with other regulators of inflammation that are induced after ECC, including the ERK pathway.

Extracellular-Signal Regulated Kinase Signaling: Inflammation and EIMD

During and following ECC, there is an increase in ERK pathway activity. ERK is part of the MAPK family, which consists of three related serine/threonine kinase groups: p38 Kinase, JNK (c-Jun N-terminal Kinase), and ERK. These kinases are ubiquitously expressed and play critical and diverse roles in proliferation, migration, differentiation, and apoptosis through transduction of extracellular signals to regulate gene expression. When an extracellular signal is received, a series of kinases are activated resulting in the translocation of ERK to the nucleus to drive

expression of a wide variety of genes. The ERK pathway is complex in that it interacts with a number of other pathways, including the other MAPKs and NFκB.

The ERKs, specifically ERK1 and 2 (ERK1/2), are the most widely studied of the MAPK family and, when over-activated, are strongly linked to unchecked cellular proliferation and cancer. The ERK1/2 pathway, like other MAPKs p38 and JNK, can be activated via phosphorylation by a variety of factors, including cytokines, mechanotransduction via the integrins and extracellular matrix, growth factors including early growth factor (EGF) and NGF, and G-coupled protein receptors (126, 164, 279, 314, 349, 369, 373). As previously mentioned, a consequence of secondary damage after eccentric exercise is the release of cytokines and chemokines (133) that recruit and stimulate the proliferation in MPCs, chemoattract and activate leukocytes, and activate the ERK1/2 signaling pathway. The ERK1/2 pathway plays a critical role in MPC activation and mobilization (83, 353). Furthermore, ERK1/2 activates downstream signaling targets that regulate muscle size such as mammalian target of rapamycin (mTOR) (205). Due to its role in MPC activation and regulating transcription of genes relating to muscle remodeling, ERK1/2 may be critical to effective repair after muscle damage.

ERK1/2 can be activated by muscle tension, including eccentric actions (209, 235, 263, 335, 369). Activation after exercise is rapid and has been shown to occur immediately and at 6h (209, 235, 263, 369), as well as at 48h after ECC (335). The early activation is primarily due to mechanotransduction and free radical signaling, as the level of ERK1/2 activation is related to the amount of muscle tension (209, 369) and is attenuated by anti-oxidant treatment during concentric contractions

(369). Enhanced activation of ERK1/2 occurs at 48h after high force ECC of the biceps muscles but not after downhill running (335), a less damaging exercise that typically does not induce infiltration of inflammatory cells (199). These results indicate that ERK1/2 is activated roughly coincident with inflammatory-mediated secondary damage following high force eccentric exercise.

Although we know little about the role of ERK1/2 and inflammation after ECC in humans, results of an *in vitro* study suggested that inflammation may be the mechanism through which ERK1/2 is activated and mediates muscle damage and subsequent healing (373). In that study, CC family chemokines stimulated ERK1/2 activation and proliferation *in vitro* and ectopic expression of these chemokines sped healing time after wounding *in vitro*. Using an *in vivo* model of skeletal muscle damage induced by cardiotoxin, they found that proliferation was prolonged when these chemokines were overexpressed. The authors theorized that the CC chemokines acted through Ras, Raf, and MEK to activate ERK1/2, resulting in increased proliferation and more rapid healing. Furthermore, ERK1/2 activity is affected by a wide variety of cytokines, including CD40L, CCL2, IL1-6, -10, -13, and -17, thereby acting as signal-transducer for a number of inflammatory pathways. Although primarily activated through pro-inflammatory cytokines and inhibited by anti-inflammatory signalers, ERK1/2 is thought to play different roles depending on the length of activation (transient versus sustained)(296). Finally, ERK1/2 is strongly implicated in the development of inflammatory-mediated hyperalgesia, although no studies have, as of yet, linked ERK1/2 with DOMS.

Molecular Inflammatory Mediators Related to Hyperalgesia (DOMS)

Changes to inflammatory signaling also are critical to the development and resolution of DOMS. Although a recognized response to unaccustomed ECC for over a century, the mechanisms behind DOMS are still poorly understood. Current theory suggests multiple contributing inflammatory mechanisms lead to enhanced sensitivity of nociceptors—specifically group IV afferents (206). Some of the signaling molecules thought to mediate post-ECC hyperalgesia include bradykinin (234), COX-2 (233), prostaglandin E2 (PGE2) (331), and nerve growth factor (NGF) (118, 234, 242). IL-6 (75, 222) and IL-1 β (28) play roles in hyperalgesia. For an additional review, the reader is referred to Hyldahl and Hubal (135) and Paulsen (269).

Recent research has implicated NGF as an important mediator of hyperalgesia. In humans, injection of NGF into the trapezius muscle increases DOMS and temporal summation of pain after ECC of the shoulder (242) and leads to progressive muscle hyperalgesia and pressure pain under non-exercise or injury conditions (118). Murase and colleagues (2010) found that ECC results in the upregulation of NGF transcripts in rat muscle, as well as sensitizing thin-fiber afferents to hyperalgesia (234). The source of NGF is not yet known, though it may be released from muscle fibers, macrophages, or other cells.

The increase in NGF may be due to bradykinin release (B2 bradykinin was also shown to trigger hyperalgesia shortly after ECC) and blocking the receptor, negating both the upregulation of NGF and hyperalgesia (234). B2 bradykinin may act by promoting the activity of COX-2, which has also been implicated in DOMS.

Attenuating or blocking COX-2 activity, which normally leads to the productions of PGE₂, inhibits hyperalgesia in part, suggesting that this may be a secondary mechanism through which hyperalgesia and DOMS develop (234, 268). PGE₂ injection also stimulates hyperalgesia (75), and levels of PGE₂ are increased after eccentric exercise (331).

Other inflammatory factors may play a role in hyperalgesia and DOMS. For example, mechanical hyperalgesia (heightened sensitivity to physical stimulus such as pressure or pin prick) IL-6 injection stimulated mechanical hyperalgesia in rats, and increased its duration after PGE₂ injection (75). IL-1 β , which may stimulate the production of NGF (96), is produced after ECC in mice (28). Blocking the binding of IL-1 β to its receptor IL-1ra, suppresses not only the production of IL-1 β but also mechanical hyperalgesia and decreases the production of MPO, muscle glutathione, spinal cord glutathione, and circulating creatine kinase activity. These findings suggest that IL-1 β enhances secondary damage and induces hyperalgesia by stimulating NGF production.

Two inflammatory signaling factors that were previously discussed (NF κ B and ERK), are also implicated in hyperalgesia. ERK is a well-established mediator of varying types of hyperalgesia including thermal, inflammatory, and mechanical hyperalgesia, as well as referred pain. Depending on the type of hyperalgesia, ERK is stimulated upstream by a variety of stimuli including integrin-mediated mechanotransduction, cytokines, and NGF (85, 335, 369, 373). Indeed, NGF-mediated mechanical hyperalgesia is thought to be regulated through ERK in non-exercise models of mechanically-induced pain (196). The activity of ERK can be

attenuated or enhanced by analgesics, such as NSAIDs and opioids (176, 260). In contrast, acupuncture is an analgesic and activates ERK. When ERK activity is suppressed so are the analgesic effects of needling, suggesting that ERK may also play a role in analgesic signaling as was as promoting hyperalgesia (262). Canonical (p65) NFκB activity, which also interacts with the ERK pathway, is similarly implicated in hyperalgesia of varying causes (111, 173, 355). This pathway is stimulated by multiple upstream factors including pro-inflammatory cytokines such as IL-1β and TNF-α (258). NFκB p65, in turn, stimulates the activity and production of proteins associated with hyperalgesia, including NGF and the COX-2 pathway (173, 368). The inhibition of the p65 NFκB pathway results in suppressed pain sensation, lending further support that p65 as a mediator of hyperalgesia (70, 330). Interestingly, Rel-B activity may act to suppress COX-2 activity (11, 218), suggesting a role of the alternative NFκB pathway in the resolution of hyperalgesia. Taken together, these data suggest a number of interrelated molecular pathways leading to the activation and resolution of hyperalgesia, although to my knowledge NFκB and ERK have yet to be specifically linked to DOMS.

At the beginning of this literature review, I presented data indicating that cigarette smokers experience increased musculoskeletal injury and prolonged healing. Inflammation following muscle injury is critical to muscle recovery, regeneration, and adaptation. The final sections of this literature review discuss the specific effects of smoking on inflammation (and the specific molecular inflammatory pathways discussed above), as it pertains to muscle damage.

Smoking as an Inflammatory Response Modifier

Altered inflammatory and immune responses and dysregulation of the inflammatory balance widely reported and are characteristic of chronic smokers (71, 81, 220, 320, 329, 348). Smokers exhibit abnormalities in their inflammatory response both at rest and in response to invading pathogens and injury (317). These alterations lead to increased susceptibility, prolonged illness duration, and impaired recovery (64, 153, 182, 253). Since smoking produces a pro-inflammatory environment, and a controlled inflammatory balance is important to effective muscle function and recovery after injury, it is possible that dysregulated inflammation in cigarette smokers places them at a higher risk for injury and suppresses recovery.

Smoking Effects on Cellular Inflammation

Smoking increases susceptibility to, and prolongs recovery from, viral and bacterial invasion (64, 153, 253) and prolongs healing after injury or surgery (163, 182). The majority of studies report that there is an increase of pro-inflammatory cytokines and chemokines in the blood and airway of smokers (17, 44, 140, 329), as well as increased white blood cell counts (101, 139, 236). Of the many inflammatory cell types that are altered in smokers, of interest are neutrophils and macrophages, due to their roles in muscle remodeling. The number and chemotaxis of circulating neutrophils is higher in smokers (32, 165, 220, 252, 305-307). Conversely, neutrophil respiratory burst activity (important for molecular signaling and

phagocytosis of debris) is suppressed by smoking (297, 307, 316). In addition, neutrophil gene expression is affected. For example, a study conducted by Morozumi and colleagues (231) found that neutrophil gene expression, including that of inflammatory cytokines IL-1 β , IL-8, and TNF- α , was repressed in smokers. Raw counts for monocytes also tend to be higher in smokers than non-smokers despite the observation that differentiated monocytes (macrophages) display impaired activity (52, 124, 217, 245, 312, 316, 320).

Compared to non-smokers, smoking produces a pro-inflammatory environment through enhanced pro-inflammatory signaling and greater cell counts. At the same time, smoking decreases the beneficial activities of inflammatory cells and prevents or delays the normal resolution of pro-inflammatory signaling. Taken together, these data suggest that, in smokers, dysregulated inflammation may impact repair after muscle damage and increase susceptibility to future musculoskeletal injury.

Smoking Effects on Muscle Molecular Signaling: NF κ B and ERK1/2

Only two published studies directly investigated the effects of smoking on skeletal muscle in healthy, non-COPD smokers. In a report by Montes de Oca (226), smokers, relative to non-smokers, exhibited smaller cross sectional area of type I muscle fibers with a trend toward the same in type IIa fibers. Petersen and colleagues (276) investigated gene expression of muscle size regulatory genes as well as protein synthesis rate in skeletal muscle of smokers, finding in them higher mRNA transcripts of myostatin and muscle atrophy F-box (MAFBx), negative

regulators of muscle size, as well as lower protein synthesis rates. These data suggest a mechanism through which smoking may impact the muscle injury response. Because data on the effects of smoking on muscle gene expression are limited, I expanded my analysis to include data from other tissues to build hypotheses on how smoking could affect expression of genes related to muscle regeneration. As detailed above, the activity of the inflammatory proteins NFκB and ERK1/2 following ECC is enhanced early after ECC. Smoking activates both of these pathways.

Both smoke extract and nicotine can induce NFκB activation in a variety of cell types (53, 56, 115, 138, 374, 385). Peripheral white blood cells exposed to smoke or nicotine responded with heightened NFκB activation in neutrophils (138), macrophages (374), and lymphocytes (115). In neutrophils, NFκB activation was associated with increased IL-8 release, suggesting NFκB may play a role in elevated white blood cell counts in smokers (138). NFκB activation, via IKK/IκBα phosphorylation, is induced by smoke extract (6), while several studies have shown NFκB activation in response to smoke that coincides with enhanced activation of the ERK1/2 pathway (56, 385). Furthermore, smoke-induced-NFκB activation was attenuated after ERK1/2 activity was inhibited, and therefore ERK1/2 may be important in NFκB activation in smokers.

Because the ERK pathway is a potential mediator of cancer development via enhanced proliferation, ERK1/2 has been studied extensively in cell culture and animal models exposed to smoke extract and components or by-products of smoke. ERK1/2 is rapidly activated on a consistent basis with treatment by nicotine (54, 91,

131, 149, 244), cigarette smoke extract (CSE) (177, 208, 232, 243), or cigarette smoke by-products acrolein (91) or benzo[a]pyrene (244), with phosphorylation peaking 15 minutes after treatment. In several studies, this phosphorylation led to cell proliferation (54, 158, 244). Smoking-induced activation of the ERK1/2 pathway may be initiated by various extracellular signaling molecules, including smoke by-products binding to nicotinic acetylcholine receptors (nAChR, for example $\alpha 7$ and $\alpha 4/\beta 2$ or β -adrenoceptors) (54, 244) and to the epidermal growth factor receptor (EGFR) (149). The downstream effects of ERK1/2 activation by smoking are variable, likely due to differences in upstream mediators and the cell types investigated. Nicotine-stimulated ERK1/2 activation in vascular smooth muscle cells led to VEGF- α release via MEK (149). CSE treatment resulted in ERK1/2-mediated release of signaling molecules such as PGE2 (via COX-2) (208) and interleukin-8 (177) in human lung fibroblasts and, in concert with NF κ B, murine analogs of TNF- α and IL-8 in mast cells (232). Huang and colleagues (131) suggested that ERK1/2 activation by nicotine may be mediated by COX-2, oxidative stress (indicated by glutathione), NF κ B, and activator protein-1 (AP-1) and tyrosine kinase. These data indicate that a feedback mechanism through which these inflammatory mediators perpetuate a pro-inflammatory environment in response to smoking.

In addition to activating the NF κ B and ERK pathways, smoking also enhances production of other hyperalgesia-related molecules including COX-2 (6, 208) and NGF (95, 104, 332, 368), although to my knowledge no data exist regarding these factors in skeletal muscle of smokers. Smokers are also more likely to experience chronic (265, 287, 386) and acute (68, 103) pain. Furthermore, the use of nicotine

by smokers enhances pain, while suppressing it in non-smokers, suggesting that smoking results in chronic alterations to gene expression related to pain (288). Hyperalgesia in DOMS is generally considered to result from inflammatory mechanisms, further supporting altered inflammation in smokers.

Taken together, the data presented here suggest alterations in gene and protein expression in smokers that could relate to muscle regeneration and inflammation. Underlying changes to these pathways in response to tobacco use could potentially suppress normal muscle protein turnover and healing after damage. Furthermore, ineffective remodeling of muscle, either during normal, everyday processes or in response to injury, could increase the risk for future muscle injury in smokers. Thus, a more complete understanding of the direct effects of chronic smoking on skeletal muscle signaling is warranted.

Summary

In this review of literature, the negative effects of smoking on musculoskeletal injury risk and healing after injury were summarized. ECC as a transient model of muscle injury was discussed, along with the roles of inflammation in repair and adaptation. Additionally, effects of ECC and smoking on inflammatory-related signaling pathways were outlined, with a focus on ERK and NF κ B.

The goal of this dissertation is to understand how smoking affects muscle damage and recovery. In the first study, I examined the effects of smoking on muscle after ECC at the gross, functional level. The second study served to elucidate specific

ECC-responsive pathways in non-smokers that could be affected by smoking during EIMD and recovery. In the final study, I directly tested the ECC response of these pathways in both smokers and non-smokers to identify smoking effects on the muscle damage response.

CHAPTER III

SMOKING DECREASES MUSCLE FUNCTION DURING ECCENTRIC EXERCISE AND INCREASES DELAYED ONSET MUSCLE SORENESS

Introduction

Cigarette smoking has wide-ranging impacts on health and is closely associated with airway diseases, such as chronic obstructive pulmonary disease (COPD) and lung cancer. Recent research suggests that smoking also has adverse effects on skeletal muscle health. Epidemiological studies have shown that smoking is associated with increased risk for musculoskeletal injury (5, 150, 157, 285, 286, 343, 363), including joint and muscle tissue injury. Smoking is also associated with a prolonged healing process after injury (3, 82, 310, 317) and increased disability (145, 157, 163, 182). The specific mechanisms driving the relationships among smoking, injury, healing, and disability are unknown. A greater understanding of the relationship between smoking and increased risk of injury may lead to the development of better prevention and intervention strategies. Because the majority of existing data supporting these associations are from epidemiological studies, identifying specific regulatory mechanisms has been difficult. In this study, I used an established, controlled laboratory model of inducible skeletal muscle damage to test specific hypotheses concerning the effects of smoking on muscle injury and recovery.

Eccentric exercise is a safe, laboratory-based model designed to induce transient muscle damage (55, 58, 246, 249). These types of muscle actions, where

the muscle is lengthening under tension, place high mechanical stresses on the tissue and typically lead to injury, as indicated by decrements in muscle function, delayed onset muscle soreness (DOMS), and the release of muscle-specific proteins (including creatine kinase and myoglobin) into the circulation. The injury is, in healthy subjects, subclinical with regard to kidney function and is typically followed by full recovery within 7-10d post-exercise, although there can be a great deal of inter-individual variability in the magnitude and timing of the damage response.

In healthy individuals, a coordinated sequence of inflammatory events is critical to effective muscle repair and is orchestrated, in part, by cross-talk between muscle and the circulatory system. Smoking status is a potential modifier of inflammation and damage, and this may increase risk for prolonged healing (3, 82, 310, 317, 357) and future injury (5, 47, 48, 150, 157, 212, 286, 343, 363). Indeed, smokers are not only at risk for acute and repetitive strain (5, 285) injury, but also chronic injury or disability (157, 182), suggesting impaired healing and recovery in these individuals. Dysregulation of inflammation is a hallmark of chronic injury and disability. Smoking can interfere with the normal inflammatory response to injury, which could in turn exacerbate damage responses and/or extend recovery time. The greater incidence of musculoskeletal injury and disability in smokers suggests these individuals may experience impaired muscle function and repair, which may in turn increase susceptibility to muscle damage and compromise healing. To yield insight into muscle healing in smokers, I compared responses to the eccentric exercise model in smokers and non-smokers—well characterized in non-smokers, but not yet studied in a smoking population.

I propose that, when exposed to a standardized laboratory-based exercise challenge, smokers would experience greater damage and impaired healing as compared to non-smokers. To directly test the effects of smoking on the muscle damage and recovery response, I measured muscle function and standard indicators of muscle damage before, during, and following standardized laboratory-based eccentric exercise and compared responses between smokers and non-smokers. I hypothesized that, compared to non-smokers, smokers would have a more pronounced damage response, experiencing: 1) greater expression of circulating stress hormones but suppressed inflammatory molecules; 2) greater creatine kinase release; 3) greater muscle fatigue; 4) more prolonged strength loss; and 5) greater soreness (both peak and prolonged) in response to eccentric exercise.

Methods

Subjects

Twenty healthy men, ages 18-35, were recruited into the study. Subjects had no known chronic or concurrent ailments, were sedentary (performed no activity above 5 Metabolic Equivalent of Tasks (METs) on a regular basis), and had not engaged in resistance training exercise of the legs or heavy lifting/lowering of materials using the legs for at least six months prior to enrolling in the study. Self-reported physical activity levels were verified using the Paffenbarger activity questionnaire (259) at the initial study visit. The use of anti-inflammatory and analgesic medications, alcohol, caffeine, and new physical activities were not permitted during the course of the study. Ten subjects were non-smokers, defined

as no history of regular tobacco use. The remaining 10 participants were current smokers who had smoked at least ½ pack (10 cigarettes) each day for the previous five years or longer with no history of other tobacco use. In addition to maintaining their normal smoking habits throughout the study, smokers smoked a cigarette approximately 10 minutes before each visit to reduce potential variability due to time from the last cigarette. All participants completed a medical history questionnaire and gave written informed consent as approved by the University of Massachusetts Amherst Institutional Review Board prior to participation.

Study Design

The study consisted of eight visits over the course of 7-11d (Figure 3.1). At the first visit, blood was drawn to test creatine kinase activity. In addition, baseline soreness was evaluated, and initial strength testing of the knee flexors and extensors was performed. Within 1-4d after visit 1, the subjects returned to the laboratory in the morning (visit 2) for repeated baseline strength testing, eccentric exercise of the knee extensors, and post-exercise strength testing. Recovery was measured from visits 3-8. Blood was collected six hours after visit 2 (visit 3). Each morning from 1-5d post-exercise (visits 4-8), subjects returned to the laboratory for blood collection, soreness assessment, and strength testing. For all morning visits (visits 1 and 4-8), participants were fasted overnight except for water. At visit 2, a standardized meal was provided that consisted of 400 kcals (approximately 55% carbohydrate, 30% fat, and 15% protein), after which all subjects were required to fast until they returned for visit 3 (6h later).

Salivary Cortisol, IL-6, and Cotinine

Saliva was collected by passive drool technique at all visits, including two morning pre-exercise samples, one afternoon pre-exercise sample, and six post-exercise samples (6h and 1-5d). Subjects were fasted for 8-12h prior to collection. All subjects refrained from any actions that might result in bleeding within the mouth, such as vigorous brushing of teeth or flossing, in the hour prior to collection. Upon entering the laboratory, subjects rinsed their mouths with water and then rested in a seated position for 10min. For saliva collection, subjects were asked to lean forward slightly, head down, to encourage saliva to pool under the tongue at the front of the mouth. A straw was provided, and subjects allowed the pooled saliva to move through the straw into a small collection tube. All samples were visually inspected for blood contamination—no samples had visual contamination. The saliva was briefly centrifuged to eliminate bubbles and shipped to a clinical laboratory for analysis of the stress hormone cortisol, cytokine interleukin-6 (IL-6), and cotinine; the latter is a nicotine derivative used to confirm smoking status (Salimetrics, State College, PA, USA).

Blood Creatine Kinase Activity and Cytokine Analysis

At all visits except the exercise visit (visit 2), blood was collected by venipuncture at the antecubital region using standard aseptic technique (one morning and one afternoon pre-exercise sample, six post-exercise samples at 6h and 1-5d). Blood was drawn after saliva collection and under fasting conditions. For

plasma isolation, blood was drawn into vacutainers containing either the anticoagulant EDTA or sodium heparin. After sitting at room temperature for 15min, whole blood samples were centrifuged at 1000Xg for 10min and the supernatant collected. The resultant plasma was stored at -80°C for later analysis. Plasma separated with EDTA was shipped to a clinical laboratory for creatine kinase (CK) activity analysis (Holyoke Hospital, Holyoke, MA, USA).

Sandwich enzyme-linked immunosorbant assays (ELISAs (R&D Systems, Minneapolis, MN, USA)) were used according to manufacturer's instructions to analyze circulating cytokines in the blood before and after exercise. Plasma separated using the anticoagulants EDTA or sodium heparin was used based on the instructions from the specific kit. Briefly, plasma samples were added to 96-well plates to which a primary antibody against the cytokine of interest was attached and samples incubated at room temperature. The plate was washed, an enzyme-linked antibody against the cytokine of interest was added, and samples incubated again at room temperature. The plate was washed and a substrate solution added. After the specified amount of time, a stop solution was added to halt the colorimetric reaction, at which time the absorbance of each well was read on a Labsystems Multiskan RC plate reader (Thermo-Fisher Scientific, Rockford IL, USA) at 450nm with a correction wavelength of 540nm. The samples were run in duplicate. The resulting averages were quantified by comparing against a set of standards run on the same plate. ELISA kits were used to test plasma levels of targets c-reactive protein (CRP), interleukin-1 β (IL-1 β), IL-6, interleukin-8 (IL-8), tumor necrosis factor- α (TNF- α), vascular endothelial growth factor-D (VEGF-D).

Isometric Strength

Muscle strength was assessed on a Biodex System 3 dynamometer (Biodex Medical Systems, Shirley, NY, USA) as described previously (137, 372). Because the knee extensors were the exercised muscle group, changes in flexion strength were not expected; however, strength of the flexors was also evaluated to test if post-ECC changes to the agonist/antagonist force output ratio differed between smokers and non-smokers. Briefly, participants were positioned in the dynamometer in a seated position and straps were placed across the chest, hips, and thigh of the exercised leg to reduce the use of muscles not under examination. The pivot point of the dynamometer arm was aligned with the lateral epicondyle of the femur. Prior to testing, the subject's relaxed leg was moved through passive range of motion by a study investigator. The dynamometer was set to 0° when the leg was fully extended at the knee, and isometric (0°/sec) strength was measured at 70° of knee flexion. Participants contracted maximally against an immovable lever for 4sec either by kicking at the knee (extension) or pulling (flexion), followed by a 60sec rest. Six repetitions were performed, alternating extension and flexion (3 repetitions each). All repetitions in a set within 10% coefficient of variation were retained for analyses.

Prolonged loss of strength (i.e. in the days following exercise) is generally considered the best indirect indicator of muscle damage incurred through eccentric exercise (360). However, early (i.e. within a few minutes) post-exercise strength loss is a combination of both fatigue due to the exertion during the exercise session

and any damage to the muscle itself incurred during exercise. Therefore, strength loss was tested in two phases: early (5min post-exercise) and prolonged (1-5d post-exercise), also referred to here as “recovery”. These data were analyzed separately for each phase.

Biodex torque (N•m) data for isometric and eccentric strength were exported and analyzed using a custom-written Matlab program (version R2009b; Mathworks, Natick, MA). To eliminate noise, a 20-Hz low-pass Butterworth filter was applied to each channel. For some contractions, there was a slight signal offset such that baseline (no torque produced) measured either above or below 0 torque; therefore, to remove the signal offset, the baseline for each contraction was manually identified using plotted data. Manual identification was then used to outline the following critical points of each contraction: 1) initiation of torque production; and 2) start and 3) stop points for the plateau phase of contraction—the phase during which maximal torque production was roughly maintained. The Matlab program used these points to identify additional variables, including: the time to peak torque, peak torque and work, and the end of contraction. This process was repeated for all contractions. These results were exported into Microsoft Excel to be organized for further analysis using SAS statistical software version 9.3.

Eccentric Exercise

The exercise consisted of 100 (10 sets of 10 repetitions) maximal eccentric contractions of the knee extensors performed on the Biodex dynamometer.

Participants were positioned as described above. The exercise leg was moved

passively to 35° of flexion by the study investigator, and participants were instructed to kick with maximal effort. The dynamometer elicited greater force against the leg than the subject could produce in resistance, causing the knee to bend and move into full flexion. The complete range of motion was ~75° for each participant and set prior to exercise using passive resistance points. Repetitions were approximately 4sec in length, followed by a 10sec rest. Subjects also rested for 1min after each 10-repetition set. After the last set there was a 5min rest period, followed by isometric strength testing (early phase). Throughout the exercise and testing sessions, the subjects were verbally encouraged to perform at maximal effort and all procedures were executed using the non-dominant leg.

The exercise session was designed to both elicit fatigue and to stress the muscle eccentrically so as to result in prolonged damage and recovery. I also collected data during the exercise (torque and work) to determine if differences in exercise-induced strength loss could be explained by differences in muscle performance during the exercise session.

Soreness

Soreness was assessed at the initial (baseline) visit and on each day from 1-5d after the exercise visit (55, 58, 246). Soreness was evaluated prior to strength testing in order to eliminate any effect of strength testing on soreness. Participants were instructed to perform two controlled one-legged squats on the non-dominant (exercise) leg from standing to sitting in a chair and back to standing, with each portion of the movement lasting approximately one second for a total of two

seconds for each squat. Upon completing the squats, participants marked a single vertical line on a 100mm visual analog scale (VAS) to indicate peak soreness during the squats. A “0” indicated no soreness, “100” indicated maximal soreness. The distance (mm) from the beginning of the line to the vertical mark was measured and recorded.

Statistical Analyses

For early strength loss, a 2-factor analysis of variance (ANOVA)(92) was used (group*exercise) for absolute values and the values normalized to bodyweight. A Student’s T-Test was used to analyze these data when expressed as percent baseline (322). For recovery visits, data were analyzed using a 2-factor (group*exercise) repeated-measures ANOVA, with repeated subject ID measures and a group*time interaction term for absolute values, those normalized to bodyweight, and when expressed as percent baseline.

For the exercise session, each statistically-analyzed point represented the average of three repetitions: baseline was repetitions 3-5 (to account for any learning or ramp up in the first few contractions) and all other points represent the average of the last three repetitions of each set. Strength values for each set were analyzed using a 2-factor (group*exercise) repeated-measures ANOVA, with repeated subject ID. The repeated-measures ANOVA was also used to analyze soreness and CK activity. Post-hoc differences were assessed using Tukey’s HSD testing. Student’s T-Tests were used to compare both total work done between

groups (345) and peak soreness. Blood and salivary measures were analyzed by 2-factor ANOVA (group*time) for baseline differences; when there were differences, baselines were included as a co-variate as appropriate in further testing. The full dataset for each analyte was tested using either a 2-factor RM-ANOVA or analysis of co-variance (ANCOVA)(group*exercise), as appropriate. Significance was set as $p < 0.05$ and all testing was performed using SAS statistical software (Version 9.3; SAS Institute, Cary, NC). All data are presented as means \pm standard error.

Results

All subjects (N=10 smokers and 10 non-smokers) successfully completed the exercise protocol and complied with study requirements. Final subject characteristics are presented in Table 3.1.

Salivary Cortisol, IL-6, and Cotinine

Salivary cotinine, cortisol, and IL-6 were measured before and after exercise. Smoking status was verified using salivary cotinine measures, collected at every visit, and corroborated that all subjects were categorized appropriately and maintained regular smoking (or non-smoking) habits throughout the course of the study (Table 2) ($p < 0.05$). Levels of cortisol differed significantly ($p < 0.05$) between the morning and afternoon baseline measures for both non-smokers (0.511 ± 0.07 and $0.209 \pm 0.04 \mu\text{g/dl}$, respectively) and smokers (0.417 ± 0.05 and 0.224 ± 0.04). This finding was expected, as cortisol presents with strong diurnal rhythmicity; cortisol levels are highest in the morning and decrease throughout the day. Therefore, the

afternoon (6h post-exercise) visit was compared to the afternoon baseline, only, and the morning measures (1-5d post-exercise) compared to the morning baseline. There were no differences between group responses, after exercise, or overall between groups when comparing between the afternoon visits or among the morning visits. IL-6 salivary measures were highly variable and levels were undetectable for a number of samples, leaving five non-smokers and nine smokers in the final analysis. Baseline levels did not differ between visits and were therefore averaged for the remaining analyses. These baseline levels were significantly higher in non-smokers (40.86 ± 10.97 pg/mL as compared to smokers, 11.85 ± 2.04), yet there was no overall effect of smoking, exercise, or interaction. However, given the number of samples eliminated because levels were below detection, these data must be given little weight.

Creatine Kinase Activity

Plasma creatine kinase activity after exercise was used as an indirect marker of acute skeletal muscle injury. The data supported the effectiveness of the exercise, as there was a significant increase in creatine kinase activity in both groups following exercise that peaked at 5d ($p < 0.05$; Figure 3.2). However, there were no significant differences between smokers and non-smokers in their response to the exercise ($p = 0.76$) or between groups overall ($p = 0.62$), denoting similar overall responses.

Plasma Cytokines

Levels of IL-8 and IL-1 β were below the level of detection and therefore could not be analyzed. For all other plasma cytokines, pre-exercise levels were similar between the two baseline visits (morning and afternoon) and were therefore averaged for all of the following analyses. There was no difference between smokers and non-smokers overall (group difference) or difference between groups in their exercise response (interaction) for any of the cytokines tested. However, levels of all four cytokines were affected by the exercise session. Plasma IL-6 was significantly higher at 6h compared to all other time points (non-smokers: $222.6 \pm 37.2\%$ baseline; smokers: $180.0 \pm 27.2\%$) and this response did not differ between groups. TNF- α levels for both groups were subtly yet significantly different between 6h and 2d post-exercise ($p < 0.05$; non-smokers: 92.9 ± 4.2 vs $104.6 \pm 5.4\%$; smokers: 91.5 ± 4.0 vs $98.4 \pm 3.5\%$). For both groups, CRP levels significantly differed from baseline at all post-exercise visits ($p < 0.05$), peaking at 1d (non-smokers: $200.6 \pm 46.9\%$ baseline; smokers: 232.6 ± 55.5). There were no significant effects of group, alone, and the interaction term did not reach significance. Levels of VEGF-D for both groups were significantly lower at 1 and 2d after exercise as compared to pre-exercise levels ($p < 0.05$; non-smokers: 93.6 ± 1.7 and $92.3 \pm 2.4\%$ of baseline; smokers: 91.9 ± 3.3 and $92.0 \pm 2.4\%$).

Isometric Strength

Baseline

Before exercise, smokers appeared to be slightly stronger for extensor strength than non-smokers ($p < 0.05$; Figure 3.3a), but this did not reach significance when normalized to bodyweight ($p = 0.13$; Figure 3.3b). Baseline flexor strength was not different between non-smokers and smokers, for absolute values (non-smokers: $95 \pm 8.4 \text{ N}\cdot\text{m}$; smokers: $108 \pm 5.8 \text{ N}\cdot\text{m}$; $p = 0.22$) or when normalized bodyweight (non-smokers: $1.28 \pm 0.09 \text{ N}\cdot\text{m/kg}$; smokers: $1.35 \pm 0.09 \text{ N}\cdot\text{m/kg}$; $p = 0.61$).

Early Strength Loss

Isometric strength was tested five minutes after completing the eccentric exercise session. This early strength loss is generally composed of temporary fatigue from the exercise plus any damage that has occurred. For extension, strength significantly decreased after the exercise for both non-smokers ($29 \pm 6.6\%$ loss) and smokers ($44 \pm 4.2\%$ loss) (Figure 3.4a; $p < 0.01$). There was no main effect of group ($p = 0.15$); however, there was an interaction between smoking and exercise such that smokers lost significantly greater strength after the exercise than did non-smokers ($p = 0.04$). When normalized to bodyweight, the significant effect of exercise remained (Figure 3.4b; $p < 0.01$), with no overall effect of smoking ($p = 0.61$). The interaction also remained significant, with smokers experiencing a greater loss of strength after exercise than non-smokers ($p = 0.04$). Finally, when expressed as a percent of baseline, there was a trend for greater strength loss after exercise in smokers (Figure 3.4c; $p = 0.07$).

For flexion, both groups lost similar amounts of strength early after exercise (not shown; non-smokers: baseline 95 ± 8.4 vs post-exercise $88 \pm 8.1 \text{ N}\cdot\text{m}$; smokers: 108 ± 5.8 vs $94 \pm 4.7 \text{ N}\cdot\text{m}$; exercise: $p < 0.01$; group $p = 0.33$; interaction $p = 0.25$). Normalizing to bodyweight did not alter results, with a significant effect of exercise ($p < 0.01$), no effect of smoking ($p = 0.90$), and no interaction ($p = 0.21$). Expressing flexion strength as a percent of baseline also did not modify results (not shown; non-smokers: $94 \pm 5.4\%$; smokers: $87 \pm 2.5\%$; $p = 0.27$).

Prolonged Strength Loss

The “strength recovery phase” consisted of visits 4-8 and spanned the time from 1-5d post-exercise. Because there was a 24h period between the exercise session and the first recovery measure, any effect of metabolic fatigue on torque production was minimized. Isometric strength at each time point was compared to baseline and between groups. For peak isometric extension torque (Figure 3.5a; $p < 0.01$), strength decreased significantly and most profoundly at 1d post-exercise (percent loss from baseline: non-smokers, 33%; smokers: 38%), with a linear increase in strength to 5d toward baseline (non-smokers, 6%; smokers 14% loss from baseline). There was no effect of smoking ($p = 0.27$) and no group*exercise interaction ($p = 0.37$). When normalized to bodyweight, the effect of exercise remained (Figure 3.5b; $p < 0.01$), with no effect of smoke ($p = 0.67$) and no interaction (0.41). As a percent of baseline, the effect of exercise persisted (Figure 3.5c; $p < 0.01$), but, again, there was no effect of smoke ($p = 0.15$) and no interaction ($p = 0.24$). These

data indicate that the exercise was effective at inducing damage, and that smokers and non-smokers exhibited similar prolonged strength loss.

As with extension, flexion peak torque during recovery had a significant effect of exercise (Table 3.3; $p < 0.01$), with no effect of smoking ($p = 0.15$) or interaction ($p = 0.35$). These patterns remained with normalization to bodyweight and percent baseline.

Muscle Function During Eccentric Exercise

Eccentric Peak Torque Loss

Eccentric peak torque was plotted at baseline and the last 3 contractions of each set. Eccentric torque decreased significantly from baseline for both groups beginning in set 4 ($p < 0.05$) and continuing through the end of exercise (sets 5-10, $p < 0.01$) in absolute values (Figure 3.6a), when normalized to bodyweight (Figure 3.6b), and when expressed as a percent of baseline (Figure 3.6c). There was no overall effect of group for absolute ($p = 0.14$), normalized ($p = 0.54$), or percent baseline values ($p = 0.1$). Smokers (26% loss) had a more dramatic decrease in eccentric peak torque from baseline to the end of exercise than non-smokers (36% loss) for absolute and normalized torque values and when expressed as a percent of baseline (interaction: $p \leq 0.01$).

Eccentric Work

Total Work

To determine if the difference in strength loss during and 5min post-exercise could be explained by differences in work done during the exercise, work was analyzed between groups. Over the course of the exercise, smokers did significantly more total work (~30% for absolute values, ~20% when adjusted for BW) than did non-smokers in absolute values (Figure 3.7a; $p=0.03$). However, when differences in bodyweight were accounted for, total work done did not differ between non-smokers and smokers, each group producing 614J/kg and 731J/kg, respectively (Figure 3.7b; $p=0.13$).

Work Capacity over Exercise Bout

While the total volume of work did not differ between groups, the pattern of work done over the course of the exercise could explain differences in the exercise response. Therefore, I analyzed work done in each set throughout the exercise session and compared these changes between groups.

Work capacity decreased over the course of the 10-set exercise session, with work during the final set at 23% and 30% loss from baseline for non-smokers and smokers, respectively (Figure 3.8a; $p<0.01$). While there were no overall differences between smokers and non-smokers in absolute values over the course of the exercise session, there was an interaction ($p<0.01$), with smokers decreasing work capacity to a greater degree than non-smokers. The effect of set and the interaction

term remained significant when work was normalized to bodyweight (Figure 3.8b; $p<0.01$) or expressed as a percent of baseline (Figure 3.8c; $p<0.01$).

Delayed Onset Muscle Soreness

As expected, exercise significantly increased soreness in both groups ($p<0.01$; Figure 3.9) that peaked at 2d (non-smokers: 41.5mm; smokers: 58.3mm). The interaction term was non-significant ($p=0.16$), as the pattern of soreness development was not different between groups after exercise. Both groups followed the pattern of DOMS typically seen in this exercise model. However, smokers reported greater soreness overall ($p=0.01$) across the entire time course post-exercise as well as greater peak soreness (non-smokers: 43.4 ± 5.5 mm; smokers: 59.0 ± 4.7 mm; $p<0.05$).

Discussion

This study tested the relationship between smoking and muscle injury by comparing responses to damage-inducing eccentric exercise. Epidemiological studies have reported greater risk for injury, disability, and prolonged healing in smokers relative to non-smokers. Given these observations, I hypothesized that smokers would have: 1) greater expression of circulating stress hormones but suppressed inflammatory molecules; 2) greater creatine kinase release; 3) greater early strength loss; 4) more prolonged strength loss; and 5) greater soreness (both peak and prolonged) in response to eccentric exercise. The findings of this study

support hypotheses 3 and 5: smokers had greater eccentric strength loss during and isometric strength loss following ECC and greater peak soreness.

Based on previously published reports linking smoking to dysregulated inflammatory signaling, I hypothesized that ECC-mediated changes to circulating levels of stress hormones and inflammatory molecules would be altered in smokers. With the expected exception of cotinine (a product of nicotine degradation) and baseline levels of salivary IL-6, levels of circulating factors tested did not differ between smokers and non-smokers. While previous studies have shown differences in some of these factors in chronic smokers at rest (17, 23, 277), the majority of studies use older smokers or those who have already developed smoking-related diseases such as COPD. It is important to note that the population of smokers in this study was young and relatively healthy; it may take a longer period of regular exposure to cigarette smoke to result in the changes to circulating factors seen in other studies.

In support of previously published literature, there was a significant increase of IL-6 after exercise (34, 112, 313). TNF- α levels were significantly lower at 6h compared to 2d (non-smokers: 11.7% difference; smokers: 6.9%). CRP levels were also affected by exercise in both groups, peaking 1d post-ECC (non-smokers: ~201% of baseline; smokers: ~233%). Alterations to TNF- α and CRP are not always found after eccentric exercise (36, 123, 199, 222, 254, 313), although some studies have reported altered levels of these analytes (42, 266, 278). Both CRP and TNF- α stimulate phagocytosis by macrophages; the results presented here indicate

potential phases of macrophage activity that support the progression through the primarily pro-inflammatory phase of the eccentric exercise response.

VEGF-D levels were suppressed slightly, but significantly, at 1 (~93% of baseline) and 2d (~92%) post-ECC. To my knowledge, this is the first study to report changes to VEGF-D levels in circulation in response to muscle damage. VEGF-D is typically associated with lymphangiogenesis, although some evidence supports its role in angiogenesis (151). It is possible that the decrease in circulating VEGF-D indicate either: a) enhanced binding of this protein, decreasing circulating (free) levels; or b) a suppression of angiogenesis during the initial stages of the muscle damage response. Recent research suggests that a single bout of eccentric can suppress angiogenesis during the initial stages of post-exercise recovery (134), though little research currently exists regarding the vascular response to eccentric exercise. Additional research is therefore required to understand how VEGF-D may impact muscle recovery.

It initially appeared that the smokers in this cohort were stronger at baseline (19% higher in smokers) and experienced greater overall loss of force production during (final set, 10% greater loss in smokers) and following exercise (5min post-exercise, ~15% greater in smokers). However, when modest bodyweight differences were taken in to account, differences between groups were no longer present for baseline strength or sustained strength loss, while strength losses during and 5min post-exercise remained significantly greater in smokers.

These data support the first hypothesis (greater early strength loss in smokers) and revealed greater muscle fatigue in smokers during the exercise. In

contrast, the second and third hypotheses were refuted. Smokers did not differ from non-smokers in their prolonged strength loss or CK activity, suggesting a suppressive effect of smoking on fatigue rather than greater overt muscle damage. The data confirmed the fourth hypothesis and I found that smokers reported higher peak soreness (~15% greater in smokers) and overall greater DOMS, which is indicative of differences in the damage response, such as dysregulated inflammation. Taken together, these data showed that smokers fatigued more with eccentric exercise and experienced great levels of soreness, but indirect markers of muscle damage other than soreness (prolonged strength loss and creatine kinase release) were not significantly affected.

Smokers experienced greater early strength loss (fatigue), coupled with a more substantial loss of force production during, and shortly following, eccentric exercise. Previous studies have shown greater muscle fatigue in response to electrically stimulated contractions following chronic smoke exposure in mice (290) and in humans (370). This is the first study to my knowledge documenting greater fatigue in smoking during and after voluntary eccentric contractions. Mechanisms that may explain this phenomenon include: 1) changes in blood flow due to smoking leading to altered clearance of metabolic by-products and/or decreased O₂ delivery; 2) suppressed oxidative metabolism (162, 290, 328); and 3) fiber type distribution differs between smokers and non-smokers, with smokers having a lower prevalence of fatigue-resistant Type I muscle fibers (169, 290, 328).

Immediately after smoking, muscle blood flow is acutely increased for a brief period (approximately 5min)(364), although smoking is conversely known to

decrease overall blood flow, more pertinently within muscle during stimulated contractions (370). In addition, previous reports have shown impairments to oxidative metabolism in individuals exposed to cigarette smoke (169, 328). However, in the study subjects were performing maximal eccentric contractions, which rely more heavily on anaerobic, rather than oxidative, metabolism. Given on these prior studies, it is unlikely that limited blood flow or suppressed oxidative metabolism contributed significantly to the greater fatigue found during and shortly following maximal eccentric exercise in the smoking group, although this was not tested directly.

While I did not examine muscle fiber types in these subjects, data in the literature support the hypothesis that differences in fiber-type distribution might explain the greater fatigue in smokers. Anaerobic (Type II) muscle fibers are more prone to fatigue than those that rely primarily on oxidative phosphorylation (Type I). A shift in fiber type distribution away from Type I and corresponding greater fatigue has been found in several studies using animal models of long-term cigarette smoke exposure (290, 328). Furthermore, Type I fibers from human smokers are smaller in both cross-sectional area (169, 226) and number (169) relative to non-smokers. These differences appear to be reversible, since fiber type distribution in former smokers was no different than non-smokers.

Fiber-type shifts in smokers away from Type I fibers may result from the more hypoxic environment caused by carbon monoxide inhalation during smoking (294, 311). In addition, nicotine and other components of smoke may influence fiber type independent from carbon monoxide exposure. Taken together, these data

suggest that smoking impacts fiber type distribution such that smokers rely more on anaerobic fibers and are therefore more susceptible to fatigue, especially since the high forces involved in eccentric exercise bias recruitment to Type II fibers. In the current study, the smokers may have been unable to sustain eccentric work production over time due to a lower percentage of smaller Type I fibers and decreased fatigue resistance, resulting in greater immediate strength loss. This would not, however, impact muscle damage and therefore prolonged strength loss was unaffected by smoking.

Prolonged strength loss (an indirect indicator of damage) did not differ between smokers and non-smokers, and both groups performed a similar amount of work overall. I found, though, that while force production remained similar between groups during the first 5 sets of the exercise session, it then decreased over the second half of the exercise session to a greater extent in smokers. While an indicator of fatigue, this force reduction also means that smokers were exposed to a lower number of relative high-force contractions (those that result in the greatest amount of strain and muscle damage (250)) later in the exercise period. Nevertheless, smokers responded to the exercise session with a similar pattern of prolonged strength loss, indicating greater muscle dysfunction proportionate to the number of relative high-force contractions when the muscle is fatigued. Therefore, while these data do not obviously point to greater muscle damage sustained in smokers (using indirect markers), they do suggest that smokers could be more susceptible to damage on a relative scale. In combination with greater fatigue, this subtle increase

in susceptibility to injury in smokers could contribute to their higher risk for injury and promote disability in smokers.

Creatine kinase activity (a biochemical marker of damage) increased after exercise but did not differ between groups after exercise. This may be due to the high intra-subject variability in CK activity following eccentric exercise (248), which makes modest effects difficult to detect. Furthermore, while CK activity is a good indicator that muscle damage has occurred, it does not correlate well with the level of muscle damage. Therefore, while counter to my hypothesis, the lack of significant differences may not be surprising.

Unlike the persistent strength loss and CK activity parameters, another indirect marker of muscle damage was found to be significantly different between groups. Smokers reported higher overall soreness (2d post-exercise, 15.6% greater in smokers) as well as peak muscle soreness (16.8% greater in smokers) compared to the non-smokers. While post-hoc differences at individual time points were not significant, modest differences at each time point may have been difficult to pick up due to high variability in soreness responses. Peak soreness and the magnitude of muscle damage generally tend to be positively correlated. Inflammation is widely considered to drive DOMS after lengthening contractions. It is worth noting that smokers are known to have dysregulated inflammatory responses to injury (reviewed in (108)). Although data from the current study did not directly address inflammation, one possibility is that greater overall and peak soreness in the smoking group after eccentric exercise may be a result of dysregulated inflammatory processes during muscle recovery.

Inflammation is highly regulated and critical to muscle regeneration (190, 221, 223). Muscle stress stimulates pro-inflammatory signaling, which is followed by anti-inflammatory signaling and then a full resolution of inflammation (336, 337). Current knowledge points to pro-inflammatory signaling stimulating the release of chemical nerve sensitizers and increased intramuscular pressure from edema, together resulting in hyperalgesia and manifesting as DOMS (135). Disruptions to the inflammatory process (such as the use of non-steroidal anti-inflammatory drugs) result in delayed healing and attenuate adaptation (190, 221, 223), indicating the importance of inflammation in muscle recovery. In this study, smokers reported higher overall DOMS without obvious signs of greater muscle damage, suggesting alterations to inflammation after eccentric exercise.

Inflammatory responses are altered in smokers after traumatic injury and surgery, variances that are thought to interfere with normal healing. Inflammation after muscle injury has yet to be investigated in smokers. At rest, smokers have greater numbers of circulating neutrophils and macrophages (32, 165, 220, 252, 305-307), but leukocyte activity is generally suppressed, including neutrophil respiratory bursts (297, 307, 316) and activity of macrophages (52, 124, 217, 245, 312, 316, 320). Leukocytes from smokers tend to be pro-inflammatory, although an abnormal prevalence of anti-inflammatory leukocyte sub-types has also been reported (71, 309). I theorize that dysregulated inflammation could interfere with normal muscle-recovery signaling, leading to greater soreness, less effective healing, and higher risk for injury. Since inflammatory responses to muscle injury in

smokers have yet to be studied, it remains to be seen if altered inflammation may explain the higher soreness found in this study.

I believe the greater soreness in smokers to be indicative of a dysregulated inflammatory response after exercise, which could lead to suppressed healing and greater future risk for injury. I also believe the similar damage sustained (indicated by prolonged strength loss and CK activity) while exposed to fewer relative high-force contractions, combined with greater peak soreness, indicate a greater susceptibility to muscle damage in smokers. The primary findings indicate two possible mechanisms behind the higher risk for injury in smokers: 1) greater fatigue in smokers may increase risk for acute injury, and 2) higher soreness may indicate dysregulated inflammation, which could interfere with normal healing and promote disability. Based on these data, I speculate that smokers may be at greater risk of injury from physical activities that are novel and impose high muscular stress, particularly to those individuals who experience high levels of fatigue and soreness. I cannot make public health recommendations based on the size of this study; however, additional study is justified regarding whether acute injury risk may be reduced by paying careful attention to muscle fatigue. Further, examination of the effect of time between bouts of exercise, allowing additional time for recovery and possible reduced risk for disability, is warranted. Greater soreness in smokers may influence these individuals against participation in stressful physical activity. Over time, lower fitness levels could lead to increased risk for health consequences associated with low physical activity such as cardiovascular disease and diabetes. Further research is needed to understand the molecular mechanisms that may

underlie greater fatigue and higher soreness in order to more effectively reduce risk for injury and promote effective musculoskeletal healing in smokers.

Tables and Figures

Figure 3.1: Study Timeline.

Over the course of 8 visits, there were 8 blood draws, maximal voluntary contraction (MVC; strength measure) testing at 7 visits, and one eccentric exercise session (visit 2).

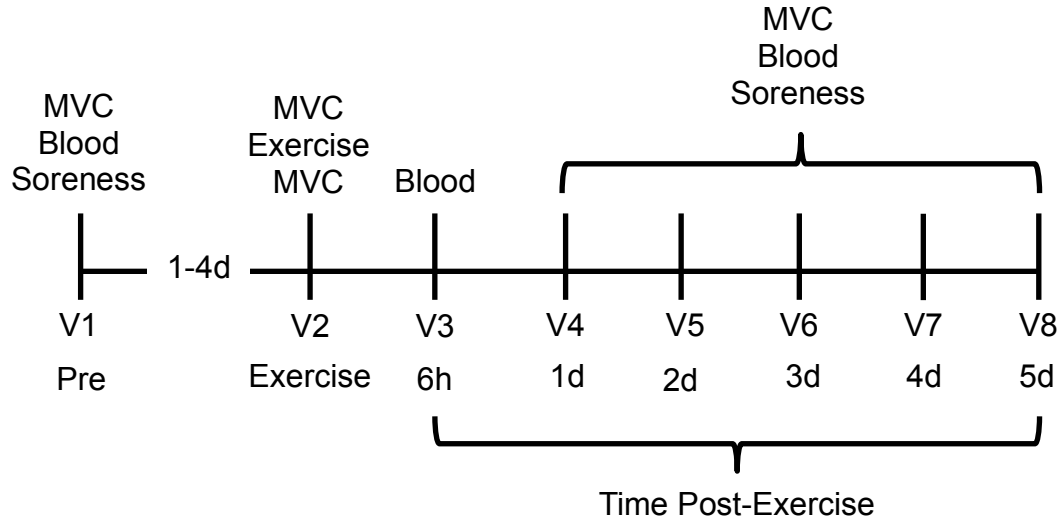


Table 3.1: Subject Characteristics.

There were no significant differences between groups for height, weight, body mass index (BMI), or age. Pack-years is a product of packs of cigarettes smoked per day and years of smoking. Data are mean \pm SEM.

	Height (cm)	Weight (kg)	BMI	Age	Pack-years
Non-smokers	176.0 \pm 1.3	74.8 \pm 4.6	24.2 \pm 1.5	25.5 \pm 1.0	N/A
Smokers	178.6 \pm 2.6	82.1 \pm 4.8	25.8 \pm 1.6	25.3 \pm 1.6	4.4 \pm 0.5

Table 3.2: Salivary Cotinine Levels.

Smoking status was verified using cotinine (ng/mL), measured in saliva that was collected at all visits. Baseline is average of V1 and V2, which did not differ. Smokers maintained their regular smoking habits throughout the course of the study. Data are mean \pm SEM. * $p < 0.05$ effect of group (smoking).

	Baseline	6h	1d	2d	3d	4d	5d
Non-smokers	1.6 \pm 0.3*	1.6 \pm 0.3*	1.2 \pm 0.3*	2.0 \pm 0.7*	1.8 \pm 0.3*	1.2 \pm 0.2*	1.3 \pm 0.2*
Smokers	308.3 \pm 44.4	355.6 \pm 72.4	326.2 \pm 67.5	265.2 \pm 45.2	291.7 \pm 57.1	315.5 \pm 65.8	351.7 \pm 67.1

Figure 3.2: Creatine Kinase.

Blood CK before and after ECC. Non-smokers —●—; smokers --□--. Data are mean±SEM. * $p < 0.05$ effect of exercise (compared to baseline). CK, creatine kinase.

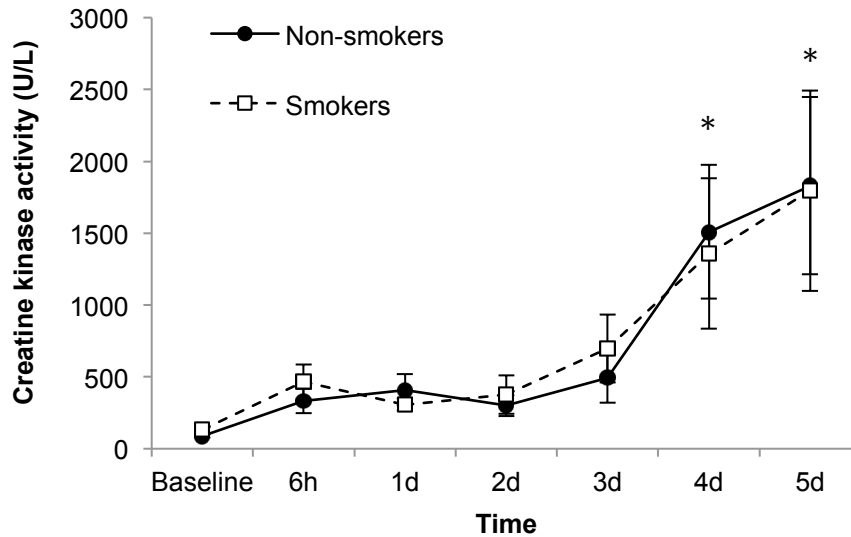


Figure 3.3: Isometric Peak Torque Extension: Baseline.

Baseline peak torque in (a) absolute values and (b) adjusted for bodyweight. Non-smokers ■; smokers □. Data are mean±SEM. * $p < 0.05$ between groups.

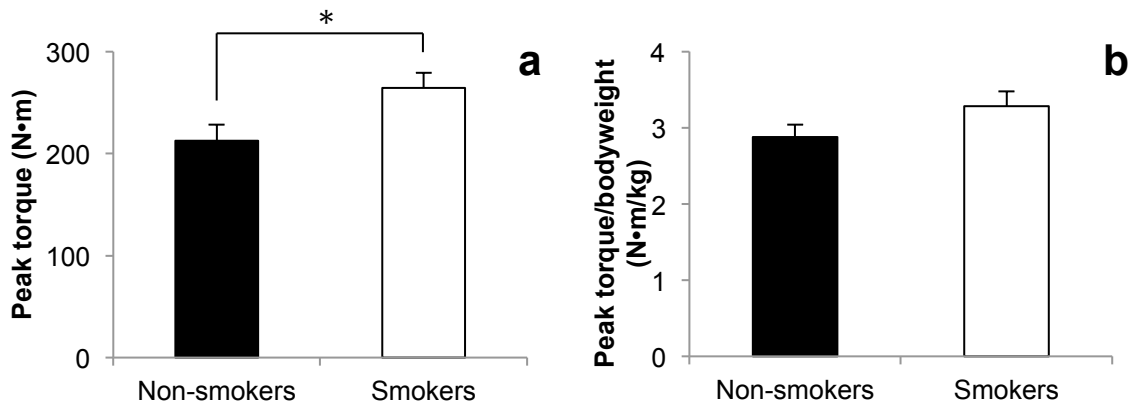


Figure 3.4: Isometric Peak Torque Extension: 5min Post-ECC.

Peak torque at baseline and 5min after ECC, expressed as (a) absolute values, (b) normalized to bodyweight, and (c) percent baseline. Non-smokers ■; smokers □. Data are mean±SEM. * $p < 0.05$ effect of exercise (compared to baseline); † $p < 0.05$ effect of group (smoking). ECC, eccentric exercise.

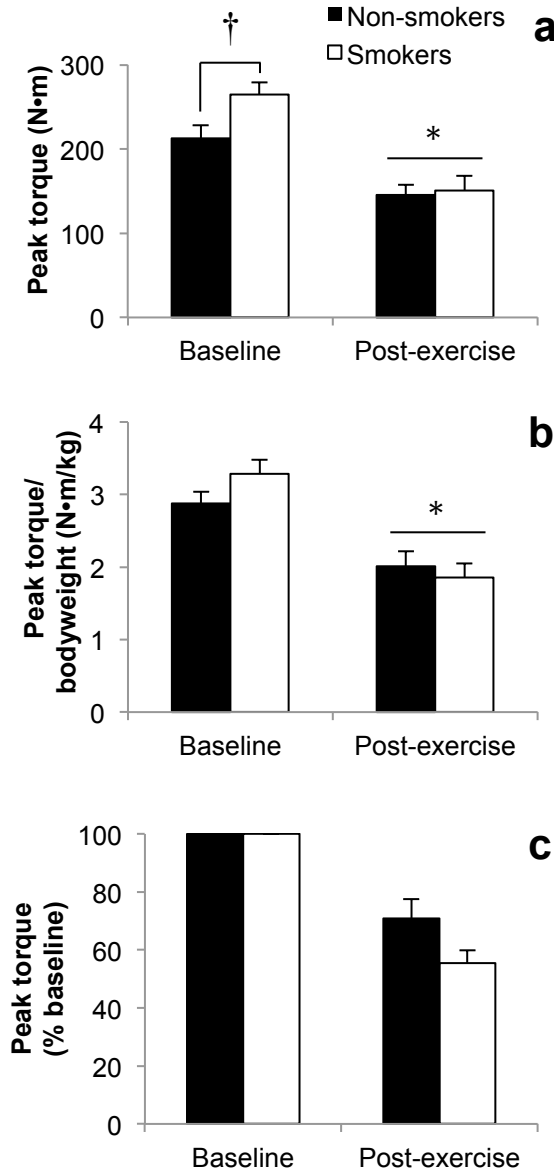


Figure 3.5: Isometric Peak Torque Extension: Recovery.

Peak torque during recovery (1-5d post-ECC), expressed as (a) absolute values, (b) normalized to bodyweight, and (c) percent baseline. Non-smokers —●—; smokers --□--. Data are mean \pm SEM. * $p < 0.05$ effect of exercise (compared to baseline). ECC, eccentric exercise.

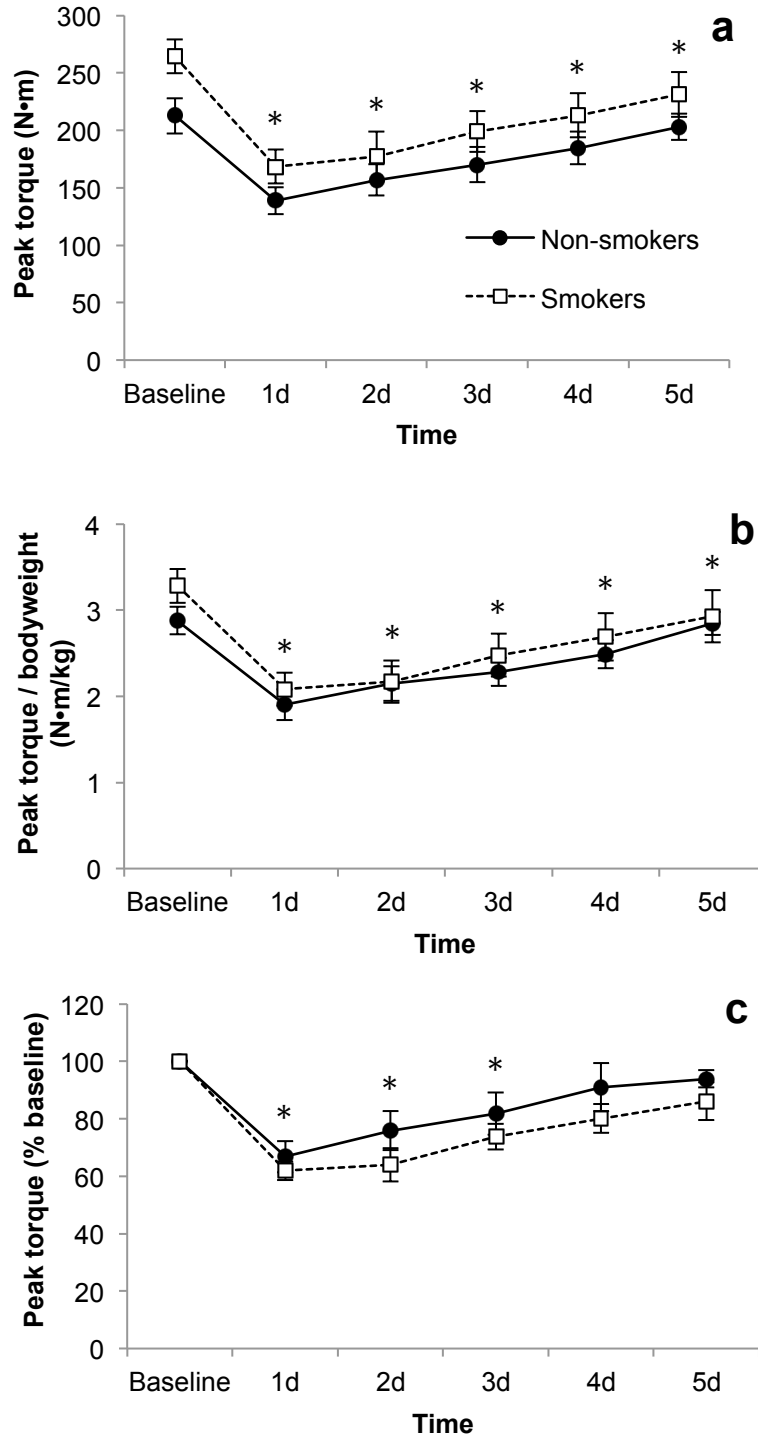


Table 3.3: Isometric Peak Torque Flexion: Recovery.

Peak torque during recovery (1-5d post-ECC), expressed as absolute values (N•m), normalized to bodyweight (N•m/kg), and percent baseline. * $p < 0.05$ effect of exercise (compared to baseline). ECC, eccentric exercise.

		Baseline	1d	2d	3d	4d	5d
Flexion torque	Non-smokers	95±8.4	90±8.8*	88±7.7	87±6.9	98±7.4	97±6.9
	Smokers	108±5.8	98±6.0*	105±6.6	109±7.3	109±6.4	111±8.1
Torque/bodyweight	Non-smokers	1.29±0.09	1.21±0.10*	1.20±0.10	1.19±0.09	1.34±0.10	1.37±0.10
	Smokers	1.35±0.09	1.22±0.09*	1.29±0.09	1.35±0.11	1.35±0.09	1.37±0.10
Percent loss from baseline	Non-smokers	0	6±2.9	7± 3.0	6±4.1	-1±6.6	-2±4.4
	Smokers	0	10±3.4	5±3.7	0±4.9	-1±3.2	-2±3.9

Figure 3.6: Eccentric Peak Torque Extension: Exercise Session.

Peak eccentric torque over the course of the exercise session expressed as (a) absolute values, (b) normalized to bodyweight, and (c) percent baseline.

Non-smokers —●—; smokers --□-- . Data are mean±SEM. * $p < 0.05$ effect of exercise set (compared to baseline); § $p < 0.05$ interaction (exercise*smoking, compared to baseline).

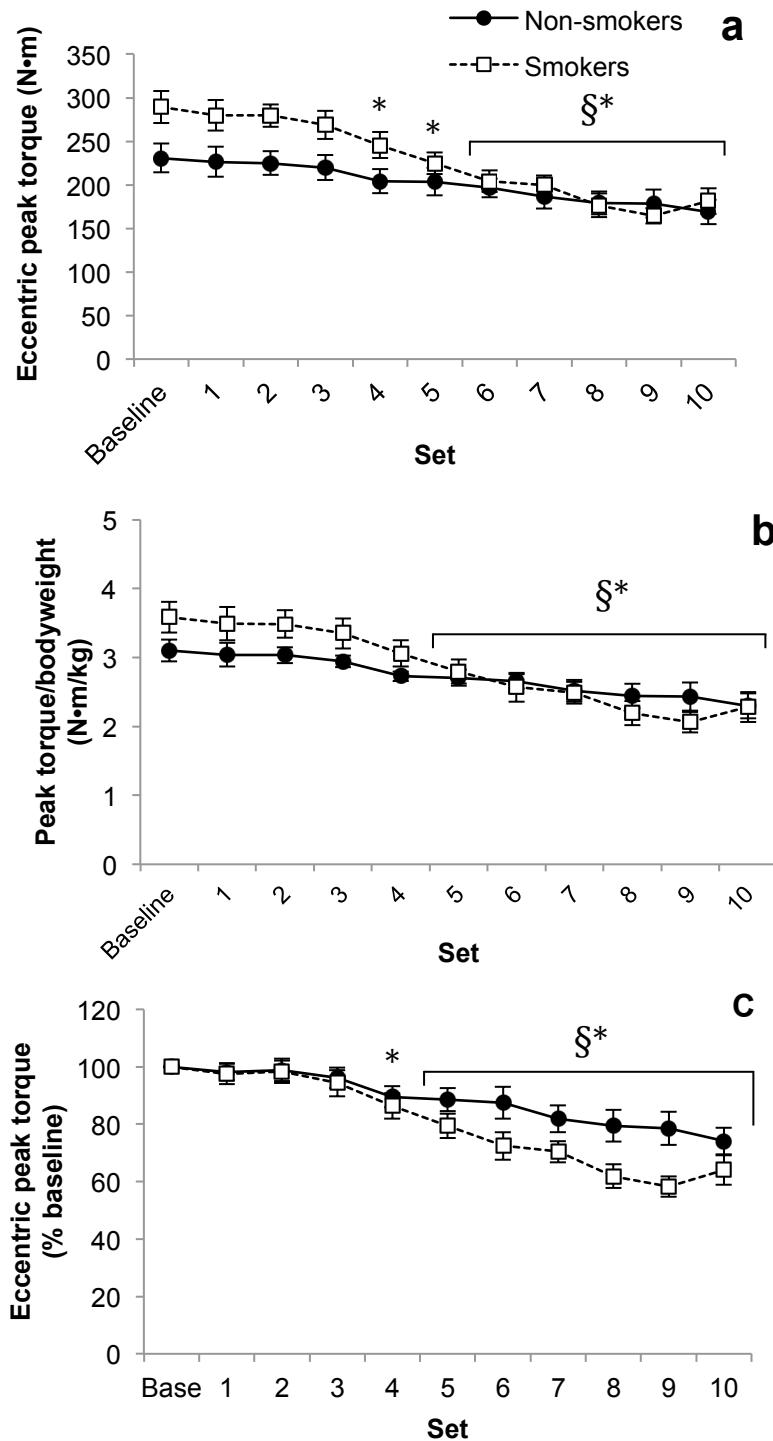


Figure 3.7: Total Eccentric Work Done: Exercise Session.

The sum of work done during the exercise session expressed as (a) absolute values and (b) normalized to bodyweight. Non-smokers ■; smokers □. Data are mean±SEM. † p<0.05 effect of group (smoking).

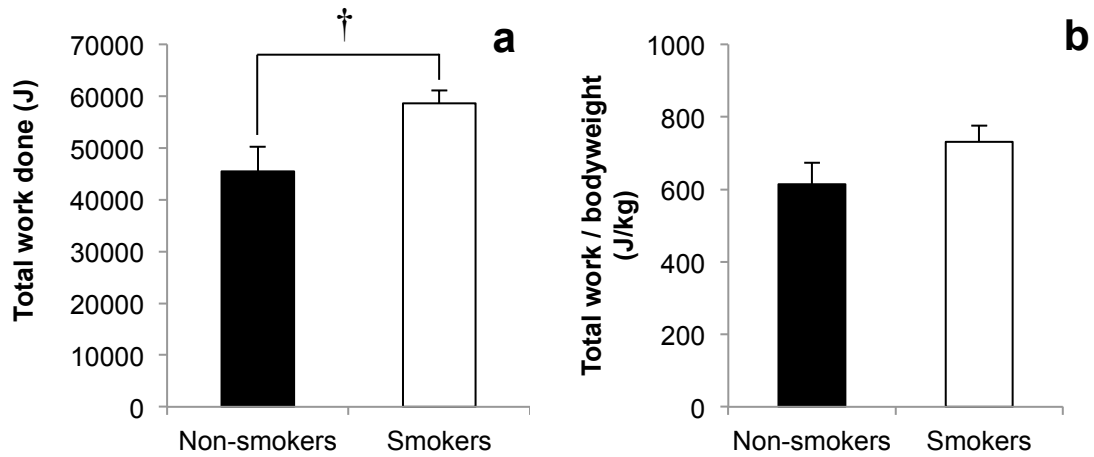


Figure 3.8: Eccentric Work during the Exercise Session.

Eccentric work done in each set over the exercise session expressed as (a) absolute values, (b) normalized to bodyweight, and (c) percent baseline. Non-smokers —●—; smokers --□--. Data are mean \pm SEM. * $p < 0.05$ vs baseline; § $p < 0.05$ interaction (exercise*smoking, response compared to baseline).

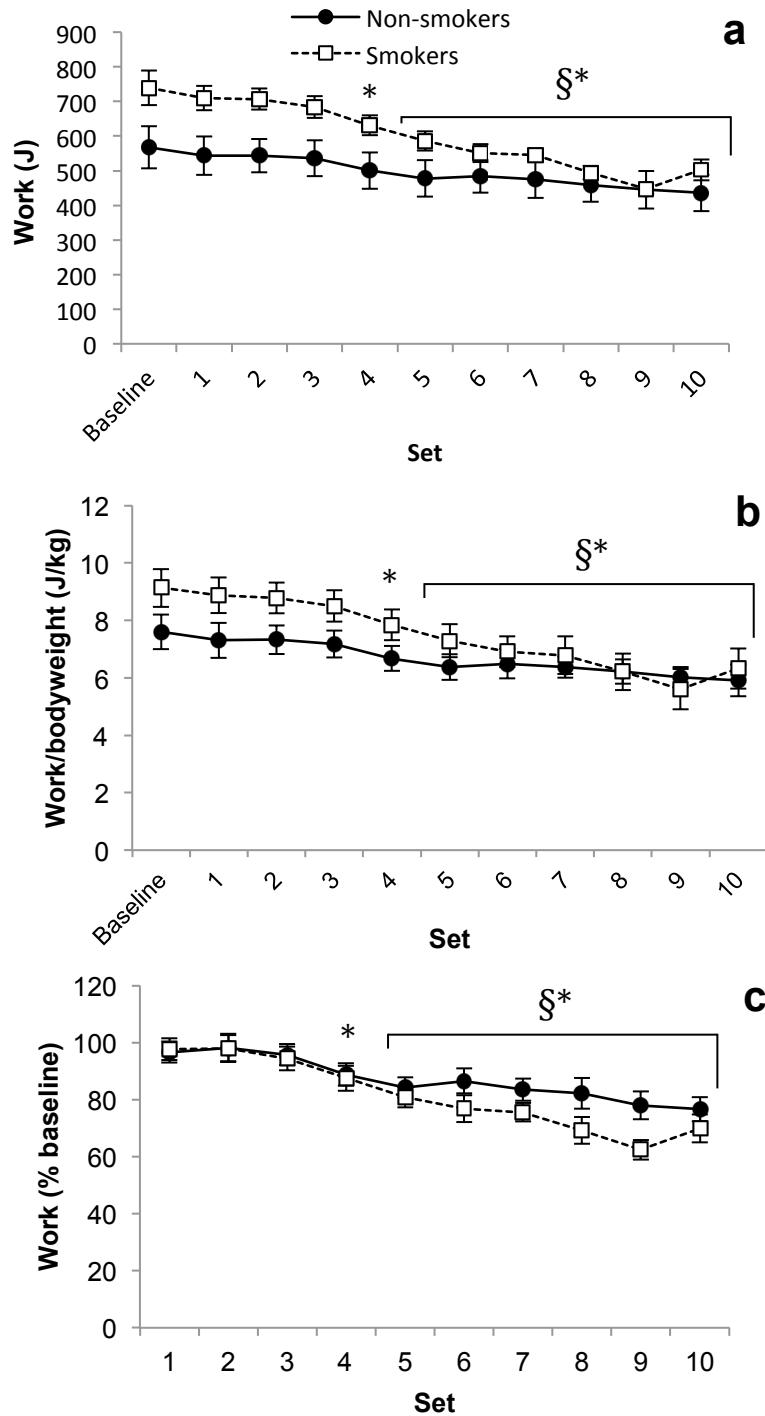
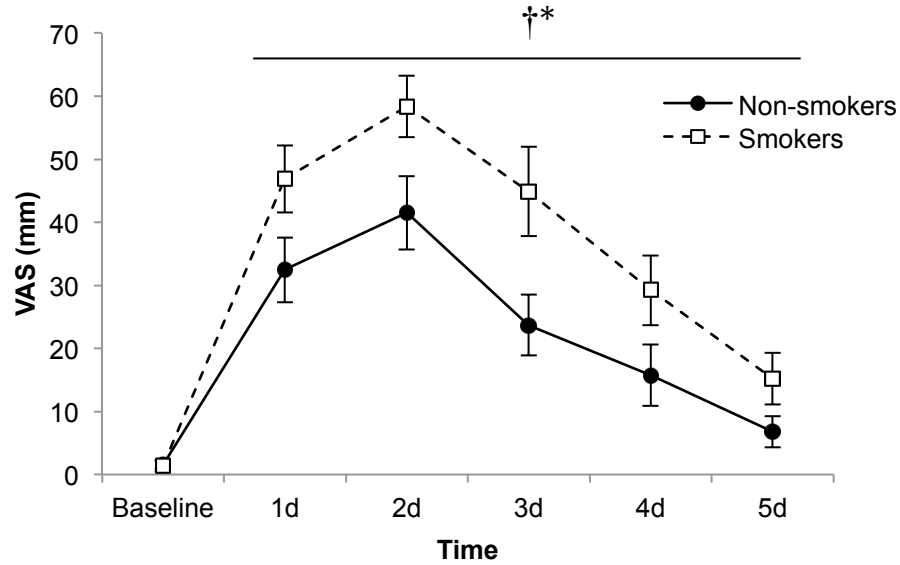


Figure 3.9: Soreness Post-ECC.

Soreness measured by VAS at baseline and 1-5d post-ECC. Non-smokers —●—; smokers --□-- . Data are mean \pm SE. * $p < 0.05$ effect of exercise (compared to baseline); † $p < 0.05$ effect of smoking (compared, overall, to non-smokers). VAS, visual analog scale; ECC, eccentric exercise.



CHAPTER IV

TRANSCRIPTOME ANALYSIS OF SKELETAL MUSCLE AT EARLY (3H) AND LATE (48H) TIME POINTS AFTER ECCENTRIC EXERCISE

Introduction

Muscle-lengthening (eccentric) contractions (ECC) stimulate a damage/repair process that can be described in three stages. Primary damage via mechanical strain during exercise is followed over the subsequent hours and days by secondary damage, caused by infiltrating inflammatory cells and free radical molecules. The final stage, repair and regeneration, can last for a week or more post-exercise. This stage overlaps somewhat with the secondary damage stage both temporally and in its underlying molecular mechanisms. Pathways implicated in early changes after ECC have been previously reported, yet mechanisms that drive secondary damage and repair are poorly understood, especially the critical shift from damage to resolution and repair/regeneration. Understanding the molecular mechanisms that mediate these processes may help reduce healing time, minimize permanent consequences to muscle such as fibrosis, and protect against future injury.

Neutrophils dominate in the early phase of inflammation following ECC (up to 2d post-exercise), where they degrade necrotic and (sometimes healthy) neighboring tissue. Yet, free radical molecules produced during this phase can also promote regeneration, for example H_2O_2 stimulates protein kinase c (PKC) activation (160). Pro-inflammatory processes include the activation of signaling

molecules such as nuclear factor kappa-B (NFκB) (reviewed in Haddad (111)) and cytokine release such as interleukin-6 (IL-6 (271, 313)) and macrophage chemoattractant protein-1 (MCP-1(133, 166, 255)). Next, M1 macrophages, which act in a similar manner to neutrophils, infiltrate and continue to promote pro-inflammatory activity. The late phase of post-ECC inflammation (roughly 2d+ post-ECC and onward) is characterized by anti-inflammatory (M2) macrophages and other signaling that quenches pro-inflammatory signaling and cell activity, which suppresses further damage and stimulating repair and adaptation. At the end of this phase, inflammation generally resolves back to baseline. If this progression of inflammatory events favors the pro-inflammatory environment, secondary damage can occur. However, effective repair and adaptation can be suppressed when inflammation is attenuated, indicating that pro-inflammatory processes are critical to muscle recovery (168, 190, 221, 223).

The molecular mechanisms controlling the balance between secondary damage and muscle repair are poorly understood, particularly during the transitional period (~2d post-ECC). ECC is an excellent laboratory model that instigates transient muscle damage, repair, and adaptation to investigate the underlying processes. Physiological changes following ECC include the loss of strength, increased muscle-derived proteins in the blood (such as creatine kinase), and delayed onset muscle soreness (DOMS). DOMS is a form of transient, mechanically-induced hyperalgesia that typically manifests in the hours after ECC, peaks 24-48h post-exercise and resolves within ~5d.

In a recent study, I found that chronic cigarette smokers experienced 25% greater DOMS than non-smokers after ECC. Smokers also have greater risk for musculoskeletal injury (5, 47, 48, 150, 157, 212, 286, 343, 363) impaired healing after injury or surgery (3, 82, 310, 317, 357), and dysregulated inflammatory signaling (32, 101, 165, 305, 306, 316, 320). DOMS is driven (in part) by inflammatory signaling, and research indicates that nerve-sensitizing factors, such as prostaglandins, bradykinin, and nerve growth factor (NGF) may be important in the development of both hyperalgesia and DOMS (233, 234). However, the specific mechanisms underlying the DOMS response have yet to be fully elucidated. Thus, before investigating the reason for heightened DOMS in smokers, it was necessary to first gain a better understanding of the molecular mechanisms mediating DOMS and the damage/repair cycle in non-smokers. Because of the complexity of the molecular pathways that regulate DOMS, and the paucity of data in the later phase of secondary damage (during which DOMS peaks), I used a genome-wide transcriptomic analysis to identify pathways likely to affect secondary muscle damage and DOMS.

Global transcriptional profiling coupled with subsequent biological pathway analysis can be used to better understand muscle damage, repair, and DOMS. From publically available gene expression profiles, I selected two robust skeletal muscle gene expression profile sets to compare early and later inflammatory phases in skeletal muscle after ECC. Molecular pathways of interest from this analysis were then validated in skeletal muscle biopsy samples from a new 48h post-ECC cohort.

In this study, I tested the hypotheses that: 1) at 3h post-ECC, gene expression downstream of related to pro-inflammatory signaling such as IL1, and IL6, and would stimulate NFκB and related pathways to promote additional pro-inflammatory signaling; 2) at 48h post-ECC, gene expression would relate to greater anti- and reduced pro-inflammatory signaling, for example increased gene expression downstream of anti-inflammatory proteins and cytokines such as IL4 and IL13; and 3) expression of genes related to hyperalgesia and/or DOMS would: increase at 3h and decrease but remain elevated above baseline/control at 48h post-ECC. These data were then used to develop a model of the possible inflammatory signaling pathways that mediate and heightened DOMS observed in smokers.

Methods

This study employed a two-phase approach, combining *in silico* transcriptome screens with targeted experimental validations. First, two publically available global gene expression (microarray) datasets were analyzed to identify important changes with eccentric exercise between the early and late phases of the post-exercise response (Transcriptome Analyses, below). The first study was performed at the University of Massachusetts Amherst and samples were collected early (3h) after exercise (referred to here as UM-3h) (137, 372). The second selected study (referred to here as MM) was performed at McMaster University and samples were collected from one pool of subjects early (MM-3h) and late (MM-48h) post-exercise (192). Biological pathway analysis (where groups of transcripts are

related by established biological function) was used to identify underlying molecular pathways affected at each time point. In the second stage of the analysis, I used qRT-PCR, PCR arrays, and protein analyses to validate changes in transcript abundance and protein activity predicted from the *in silico* analyses. These analyses were performed on two sets of samples: 1) a sub-cohort from UM-3h, referred to here as “Validation-early” (VAL-3h); and 2) from a new cohort in which samples were collected at 48h (late) post-exercise, referred to here as “Validation-late” (VAL-48h).

Subjects

Healthy young men participated in two similar exercise paradigms from which muscle tissue was obtained for the microarray studies, the details of which have been previously reported and are discussed below (137, 191, 192, 372). For transcript and protein validation (VAL-48h), I used a new group of subjects. For all studies, subjects were free of disease or disorder that might affect the study results, did not take any medications that might affect the result of the study as determined by a study physician, and agreed to refrain from muscle treatments, such as ice and massage, during the duration of the study. All subjects were non-smokers, and none had participated in resistance training of the legs in the six months prior to enrolling in the study. In UM-3h, there were 35 sedentary men ages 18-30. VAL-48h was comprised of 9 sedentary men ages 18-35y. Interviews were used to verify that the activity level of both subject pools was below 5 Metabolic Equivalent of Task units (METs) during all activities in which subjects regularly participated. Subjects in UM-

3h and VAL-48h were allowed acetaminophen after the muscle biopsies, and were required to refrain from the use of alcohol and new physical activities until they completed the study. MM included 9 recreationally active (performed less than 3h of exercise per week) men, ages 18-25. Before each muscle biopsy, subjects in the MM study were required to refrain from exercise for 72h; alcohol for 48h; caffeine for 12h; and were requested to consumed their habitual diet for 48h. All participants gave written informed consent as approved by the University of Massachusetts Amherst Institutional Review Board (UM-3h and VAL-48h) or the Research Ethics Board of McMaster University (MM) prior to participation.

Study Design

A simplified outline of the study design is presented in Table 4.1, with details provided in the text below.

Transcriptome Analyses

UM-3h Study Design

Gene expression profiles were generated from skeletal muscle at 3h post-exercise for the UM-3h study, the details of which have been reported previously (137, 372). In addition to muscle biopsies, muscle soreness, blood creatine kinase activity, and strength loss were also measured as damage markers (137, 372). Briefly, UM-3h consisted of seven visits over the course of 8-11d (Table 4.1). At the first study visit, participants came to the laboratory in a fasted state (no food or beverage except water for 8-12h), blood was drawn, and subjects underwent a

familiarization session on the Biodex System 3 dynamometer (Biodex Medical Systems, Shirley, NY, USA), followed by baseline strength testing of the knee extensors of the exercised (non-dominant) leg. Within 2d, subjects returned to the laboratory in a fasted state and then ingested a standardized meal consisting of 400 kcals (approximately 55% carbohydrate, 30% fat, and 15% protein). Baseline soreness and muscle strength were assessed, followed by the exercise session and underwent strength testing (0h post-exercise). Three hours after the exercise, vastus lateralis biopsies were taken from the exercised and non-exercised (control, dominant) legs. On each of the following 5 days, participants returned to the laboratory to have blood drawn for creatine kinase (CK) activity analysis, soreness assessed, and strength measured.

MM Study Design

This study was used to generate gene expression profiles at 3h and 48h post-ECC. The details of this study have been reported previously (191, 192). In brief, this study consisted of four visits over the course of approximately 10d (Table 4.1). The first visit served to familiarize subjects to the Biodex System 3 dynamometer, an initial control muscle biopsy (non-exercised left leg) was taken, and blood was collected for CK analysis. All muscle biopsies were collected from the vastus lateralis and were taken from two distinct anatomical sites at least 6cm apart. Eight days later, subjects returned to the lab for the exercise visit. At this visit, a second control biopsy was collected (non-exercised leg, control sample used in the current study) followed by the exercise. Three and 48h hours later, a muscle biopsy was collected

from the exercised leg, as well as blood for CK analysis. At all biopsy visits, subjects came to the laboratory fasted and 2h prior to the biopsy consumed a standardized meal consisting of 350 kcals (approximately 57% carbohydrate, 28% fat, and 15% protein). Subjects also consumed a standardized 235mL defined formula diet (Boost®) 2h before the exercise.

VAL-48h Study Design

To confirm and extend findings from the transcriptomic analyses, skeletal muscle samples from VAL-3h and VAL-48h were tested using mRNA and protein analyses. Subjects in the validation cohort, VAL-3h were all subjects in UM-3h and did not differ significantly from the full UM-3h cohort for subject demographics or ECC response. VAL-48h consisted of six visits over the course of 13-15d (Table 4.1). Briefly, once enrolled in the study, subjects performed a familiarization session with a Biodex dynamometer, which was later used for strength testing and exercise. Visit 2, 2-4 days later, consisted of strength testing of the knee flexors and extensors, eccentric exercise of the knee extensors, and post-exercise strength testing. Two days after the exercise session (Visit 4), subjects returned to the laboratory for muscle biopsies. Strength testing was repeated at 1, 4, and 9d post-exercise (1d pre-biopsy and 2 and 7d post-biopsy; Visits 3, 5, and 6, respectively).

Eccentric Exercise

In each cohort (UM-3h, MM, and VAL-48h), subjects performed maximal eccentric exercise (ECC) of the knee extensors on a Biodex dynamometer. Subjects

were verbally encouraged to exert maximal effort through. For exercise and strength testing, UM-3h, subjects were randomized to use either their dominant or non-dominant leg, while subjects in VAL-48h used their non-dominant leg only. All subjects performed 100 (10 sets of 10 repetitions) contractions with the eccentric resistance set at 30°/sec. The other leg served as a control and did not perform the exercise. Each repetition was approximately 4sec in length with a 75° range of motion, was followed by 10sec of rest, and a 1min rest followed each 10-repetition set. In MM, subjects performed the exercise at a more rapid pace (120°/sec) and did 150 (15 sets of 10 repetitions) contractions using the right leg only (regardless of leg dominance). Each repetition was approximately 1sec in length with a 60° range of motion, performed continuously within each set (no rest between repetitions), and each 15-repetition set was separated by 1min of rest. Additional information on both studies has been previously published (137, 192, 372).

Strength Loss, Creatine Kinase Activity and DOMS

Subjects performed strength measurements on the Biodex prior to and following the exercise for UM-3h (5min, 1, and 2d post-ECC) and VAL-48h (5min and 1d post-ECC) to verify the exercise caused muscle damage. Details for UM-3h have been previously reported (137, 372). For both UM-3h and VAL-48h, subjects sat in the Biodex with the exercise leg strapped in to prevent extraneous movement. Subjects performed maximal isometric (0°/sec) contractions that were four seconds in length, three each for extension and flexion, with a minute of rest between contractions.

In each cohort, subjects had blood drawn and tested for creatine kinase (CK) levels as an indicator that the exercise had elicited damage. Blood was collected prior to exercise, at 1d (UM-3h and VAL-48h) and 2d (UM-3h and MM) after ECC. CK activity was analyzed using an ELISA-based activity assay.

Post-exercise soreness (previously reported (137, 372)) was assessed in UM-3h and peak soreness over the course of the study was used in the current study for correlation analyses with biochemical data. Soreness was not evaluated in VAL-48h. To evaluate muscle soreness prior to and on days 1-5 post-ECC, subjects performed two one-legged squats, from standing to sitting in a chair then again to standing, at a controlled pace (approximately 1s each portion of the movement) using the exercise leg, only. Subjects then marked a single, vertical line on a visual analog scale (100mm) corresponding with their amount of perceived peak soreness—no soreness was indicated by a “0” and maximal soreness was “100”. The distance (mm) from 0 provided a semi-quantitative measure of soreness.

Muscle Biopsies

Biopsy procedures were similar for all cohorts and have been reported previously (137, 193). Briefly, Bergström percutaneous needle biopsies were obtained from the vastus lateralis under local anesthetic (lidocaine) with suction. Approximately 100-150mg of tissue was collected at each biopsy site. Once removed, fat and connective tissue was dissected from the skeletal muscle, the samples were flash-frozen in liquid nitrogen, and stored at -86°C until processed. A total of two biopsies were collected from each subject in UM-3h and VAL-48h (one

from each leg—both collected at the post-exercise visit) and four in MM (two from each leg—control samples collected prior to exercise). Control samples were collected from the non-exercised leg and experimental from the exercised leg (both studies). For MM, the second pre-exercise biopsy was used as the control sample for microarray analysis.

Transcriptome Analyses

RNA Extraction and Microarray Hybridization

For the two microarray studies, total RNA was isolated using standard TRIzol extraction protocols (Invitrogen, Carlsbad, CA). For UM-3h (137, 372)), the Oligotex mRNA Midi kit (Qiagen, Valencia, CA) was used to isolate mRNA. The Ovation Pico WTA system (NuGen, San Carlos, CA) was used to create double-stranded cDNA and subsequent cRNA. Finally, Cy3-labeled cRNA (1.65ug) was hybridized to Agilent Whole Genome microarrays (Agilent, Santa Clara, CA). All procedures were performed at Gene Logic (Gaithersburg, MD) according to standard manufacturer procedures. This system allowed for the testing of ~41,000 genes. Raw data from the Agilent system was output as “txt” files for import into analysis software. Gene expression changes were filtered by p-value ($p < 0.01$) and fold change.

For MM, extracted RNA was treated with DNase I (Ambion Inc., Austin, TX) and then analyzed for purity on a Nanodrop spectrophotometer and an Agilent Bioanalyzer Nano Chip System. Samples that passed purity testing were then hybridized (850ng biotin-labeled cRNA) according to manufacturer’s instructions to Human Ref-8 BeadChips (Illumina). This system allowed the testing of ~23,000

genes, and the results were obtained using a BeadStation array reader (Illumina). The signal was calculated using GenomeStudio (Illumina), and the raw data (in Excel format) was transferred directly from a study investigator at McMaster University. Further details can be found in a previous report (192). Gene expression changes were filtered by p-value ($p < 0.01$).

All microarray results were re-analyzed from the original raw data files (downloaded from the GEO website) to standardize the analysis methods across studies. The raw data were log transformed and then evaluated by analysis of variance (UM-3h) and repeated measures analysis of variance (MM) with Partek Genomics Suite software (6.13.1106). A Fisher's Least Significant Difference test was used as the contrast method to compare between samples for UM-3h (control and exercise) and among three time points for MM (baseline v 3h, baseline v 48h, and 3 v 48h). For MM, the second control leg biopsy (collected at the "exercise" visit) was used as the baseline sample. The datasets were then filtered for significance as described above and imported into the Ingenuity Pathway Analysis software. Datasets were not filtered by False Discovery Rate to allow more robust sets to be analyzed by pathway analysis, which naturally filters out false positive results (as random false positive results would not be biologically linked to the bulk of the true results).

Biological Pathway Analysis

Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood City, CA; v 18841524) analyzed gene expression patterns to identify functional

pathways altered following ECC and create a theoretical model to explain differences between early and late exercise responses. IPA queries genes from an imported dataset for inter-relationships with other genes and functional pathways using existing literature. Using Fischer's exact test, IPA determines if the probability of association between the uploaded genes and existing cellular and molecular functions is due to chance.

I investigated genes, functions, and pathways that: 1) were shared between datasets at the 3h time point; and 2) differed between time points (3 and 48h). To do this I first performed individual analyses on the three microarray datasets (each time point for all studies—UM-3h, MM-3h, and MM-48h). Next, I performed a comparison analyses on UM-3h and MM-3h to determine similarities and differences in the 3h response. Finally, I performed a comparison analysis on all three datasets to determine similarities and differences between time points. To more specifically identify important and novel functions, pathways, regulators, and genes for additional testing, I used four primary tools from IPA: 1) Canonical Pathways compares uploaded datasets to previously defined and classically characterized elements in defined signaling pathways; 2) Diseases and Bio Functions compares the uploaded dataset to defined functional categories or known groups of disease-related genes; 3) Upstream Regulators identifies upstream regulatory molecules (ie., proteins, RNAs) that map to multiple uploaded genes; and 4) Regulator Effects uses predicted changes in regulators common to multiple genes in the uploaded dataset to predict functional outcomes downstream. These tools categorize and organize the data to help identify overarching, important, novel, or

dysregulated themes and relationships between signaling and functions throughout the datasets. The primary determinant criteria for further investigation were z-scores, which indicate the consistency and directionality of the predicted relationship, and p-value (significance). Specific numerical criteria (Table 4.2) were used to filter results from the above tools. Slight differences in cut-off values within specific criteria are due to the size of the datasets.

The final, filtered data was used to create a theoretical model to help explain the differences between the early (3h) and late (48h) secondary damage phases and mechanisms that may underlie the development and resolution of DOMS. This model was further explored with focused RNA (polymerase chain reaction) and protein analysis.

Validation Studies

RNA Analyses

RNA isolation and cDNA synthesis for VAL-3h were described above. For VAL-48h, total RNA was extracted using the standard TRIzol protocol, the quality and quantity of which were tested, in duplicate, using spectrophotometry (Nanodrop, Wilmington, DE). Samples were diluted in TE buffer (pH 8.0) and quality was assessed using the 260/280 ratio with an optimal ratio above 1.8—an additional clean up step was performed using the RNeasy kit on any samples below 1.5, which raised ratios above the threshold. mRNA was isolated using the RNeasy RNA isolation kit (Qiagen, Valencia, CA) according to manufacturer's instructions. The mRNA (0.5ug) was then transcribed to cDNA using manufacturer's instructions.

For qRT-PCR, cDNA was synthesized using the RT² First Strand cDNA synthesis kit, primed with random hexamers (SABiosciences, a part of Qiagen, Valencia, CA). For PCR arrays, cDNA synthesis was performed with a RevertAid First Strand cDNA synthesis kit, also primed with random hexamers (Fermentas, Thermo Scientific).

PCR Arrays

PCR array analysis was used to filter findings from the transcriptome analyses to extend these to 48h post-ECC. A custom-designed PCR array was used to analyze expression differences between control and exercise legs for 44 genes of interest in the VAL-48h cohort. These genes were chosen to investigate categories of protein function that were hypothesized to differ between time points based on previous studies in muscle and the microarray data from UM-3h and MM: apoptosis, cell cycle, hyperalgesia, inflammation, muscle structure, myogenesis, nitric oxide signaling, and vascular health/angiogenesis (27, 55, 65, 133, 136, 148, 190, 335, 346, 369).

Because of the large number of genes of interest, this assay was performed as a multiplex using a single well per gene and so was considered to represent a secondary screening tool. Therefore, genes chosen from transcriptome analyses for full validation and model extension were not included here and instead were tested directly via qRT-PCR. After it was combined with the RT² SYBR Green qPCR master mix, cDNA (25ng final concentration) was added to a microplate containing primer sequence sets (10uM concentration) to each gene of interest. The average threshold cycle (C_t) was determined after 40 amplification cycles on a MX3000p Real-Time

PCR System (Stratagene, La Jolla, CA, USA) and normalized to the average of 2 housekeeping genes that are not affected by exercise: β -actin and β -2-microglobulin (B2M) (193). The resultant ΔC_t for the exercise leg was then subtracted from the ΔC_t for the control leg, yielding $\Delta\Delta C_t$. This value was transformed into fold change for visualization purposes.

qRT-PCR

qRT-PCR analysis was used on samples from VAL-3h and VAL-48h to both confirm microarray findings from IPA analyses and to further extend the understanding of which pathways were altered at 48h post-exercise. Due to limited sample availability, only mRNA from a sub-set of subjects was used from VAL-48h (five subjects) studies. Prior to qRT-PCR analysis, primers were tested and the appropriate product size confirmed using 2% agarose gel electrophoresis and ethidium bromide staining. SsoFast Evagreen master mix (Biorad, Hercules, CA) was used for all PCR analyses to measure the relative levels of TNF receptor-associated factor 6 (TRAF6), NF κ B inhibitor-alpha, also known as I kappa B-alpha (NFKBIA/I κ B), and nerve growth factor (NGF). cDNA (25ng/uL final concentration) samples from control and exercised muscles were amplified in triplicate for each gene of interest on 96-well plates. No template controls were also processed. Melting curves were generated and analyzed for primer dimers following each reaction; no primer dimers or abnormalities were found in the melting curves of any of the reactions. The average of the triplicate cycle thresholds (C_t) values was used for time point comparisons with the $\Delta\Delta C_t$ method. All C_t values were normalized to

the housekeeping gene β -2 microglobulin (B2M), which has been validated to remain unchanged after eccentric exercise (193). Primers for genes of interest are shown in Table 4.3.

Nuclear Protein Extraction and NF κ B Activity Assay

NF κ B activity was tested via DNA-binding activity in the VAL-48 cohort and compared with prior measures from the UM-3h cohort. Nuclear protein was extracted by homogenizing muscle samples in low-salt lysis buffer (10uM HEPES, pH 7.6; 10mM KCl; 1.5mM MgCl₂; 0.1mM EDTA; 0.1mM DTT; 0.5M PMSF; 50uL protease inhibitor cocktail; and 0.5mg/ml benzamidine). This slurry was put through two freeze/thaw cycles in an ethanol/dry ice bath and a water bath at 37°C, mixed briefly by vortexing, and then centrifuged for 3min at 670 x g at 4°C. Cytosolic proteins (supernatant) were removed and stored. The remaining nuclear pellet was resuspended in a high-salt lysis buffer (20mM HEPES, pH 7.6; 420mM NaCl; 1mM EDTA; 25% glycerol; 1mM DTT; and 5uL protease inhibitor cocktail). After 30min incubation on ice, the samples were centrifuged at 4°C for 5min at 12,560 x g. The resultant supernatant with nuclear proteins was removed and stored at -80°C until later protein quantification with a standard BCA assay (Pierce, Rockford, IL, USA).

NF κ B DNA binding activity of nuclear proteins was analyzed using a commercially available ELISA-based TransAM NF κ B p65 assay (Active Motif, Carlsbad, CA) per manufacturer's instructions. All procedures were performed in duplicate at room temperature, with the average of the two values used for statistical analyses. Briefly, nuclear extracts (5ug of protein) were incubated for 1h

in wells containing a consensus binding sequence for NF κ B (5'-GGGACTTTCC-3'). After washing, the same wells were incubated for an additional hour with a primary antibody against the p65 subunit. The wells were washed again, incubated with a horseradish peroxidase conjugated secondary antibody for 1h, washed, and subjected to a colorimetric reaction through the addition of a developing solution for 5min followed by the addition of a stop solution. A multiwell microplate reader (FLUOstar Optima; BMG Labtech, Offenburg, Germany) was used to measure the absorbance of the plate at 450nm with a correction wavelength of 655nm.

Statistical Analyses for Validation Studies

qRT-PCR, PCR array, and NF κ B activity assays were analyzed for significant differences with exercise and over time using a 2-factor (group*exercise) repeated-measures analysis of variance (RM-ANOVA (92)). For PCR array, qRT-PCR, and NF κ B activity analysis, the raw data were analyzed with SAS statistical software (Version 9.3; SAS Institute, Cary, NC). *Post-hoc* analysis was performed using Tukey's HSD (345). Pearson product-moment correlations were performed using SAS (274). Data are presented as means \pm standard error, and significance was set at $p < 0.05$. FDR was performed on PCR arrays to account for multiple testing, resulting in more stringent, adjusted significance criteria ($p < 0.002$).

Results

All subjects in these studies conformed to their respective study requirements and completed the exercise protocols successfully. Subject

characteristics are presented in Table 4.4. There were no significant differences between study groups in terms of participant height, weight, body mass index (BMI), or age.

Strength Loss, Creatine Kinase Activity, and DOMS

Isometric strength was tested before and after exercise in UM-3h and VAL-48h, but not in MM. These data have been previously reported for UM-3h (137, 372), with subjects experiencing an average of 30.1% and 42.1% strength loss at 5min and 1d post-ECC, respectively ($p < 0.05$ vs pre-ECC). Subjects from the sub-cohort VAL-3h sustained 31.6% loss at 5min after exercise and, at 1d, 48.9%. For VAL-48h, average strength loss was 30.4% and 22.2% ($p < 0.05$) at 5 min and 1d post-ECC, respectively.

Plasma creatine kinase (CK) activity after exercise was previously reported for UM-3h (137, 372) and MM (191, 192), with significant increases in each study in response to exercise ($p < 0.05$). The pre- and post-exercise CK activity among studies was comparable. Before exercise, CK activity levels were 168.2 ± 22.2 for UM-3h and 141.3 ± 31.4 for the sub-cohort VAL-3h, 104.1 ± 52.4 for MM, and 150.4 ± 36.8 U/L for VAL-48h. After exercise, CK activity increased at 1d to 529.9 ± 77.7 (UM-3h), 439.3 ± 104.1 (VAL-3h), and 477.3 ± 85.4 (VAL-48h), and was elevated at 2d with values of 533.0 ± 135.5 (UM-3h), 329 ± 56.5 (VAL-3h), and 489.3 ± 193.2 (MM). Together, these data support that each exercise was effective at eliciting moderate amounts of muscle damage in all studies.

Biological Pathway Analysis

In UM-3h, 4,311 identifiable genes met the inclusion criteria ($p < 0.01$ and ≥ 1.2 -fold change) for significant differential expression and were analyzed using IPA, of which 1,748 were upregulated and 2,601 downregulated. For MM-3h, 1,952 genes, of approximate equal distribution between up and downregulation (963 up, 998 down) met the inclusion criteria ($p < 0.01$), 1,433 of which differed significantly in their expression from MM-48h (758 up, 680 down). There were $\sim 8\%$ fewer genes (1,792 genes) that were differentially expressed in MM-48h than in MM-3h, with a skewed distribution toward greater upregulation ($\sim 25\%$: 1025 upregulated, 767 downregulated). Of these genes, 1,349 differed significantly in expression from MM-3h. Biological pathway analyses identified a much greater breadth and number of functional and signaling changes at 3h than at 48h. In the following sections, I present overarching themes (related functions, pathways, and regulators) that were most strongly indicated in the ECC response—at 3h, 48h, shared between time points, and those that contrasted in response between 3 and 48h. The full data that passed inclusion criteria (above and Table 4.2) are shown as heat-maps in Tables 4.5-6, arranged by overarching functions for each time point in Figures 4.1a (3h) and b (48h).

Early (3h) Signaling Post-ECC

Unless otherwise indicated, numerical data presented in text below are the average z-scores of multiple, related functions or pathways from the two 3h datasets (UM-3h and MM-3h). Positive z-scores (indicate the directionality and

consistency of a predicted relationship) indicate an increase in activity/expression and negative z-scores indicate a decrease—the farther from zero, the more consistent the relationship and less likelihood that the predicted relationship was identified by chance.

At 3h post-ECC, z-scores were enhanced for functions related to cell survival and growth, specifically: 1) enhanced proliferation; 2) decreased cell death; 3) greater cell migration; and 4) greater cell survival (Figure 4.1a). Enhanced cell migration was indicated by specific sub-functions such as cell migration, movement and spreading. Various cell types were associated with these functional sub-categories including fibroblasts, connective tissue, and smooth muscle cells. Cardiovascular development and function was also enhanced, which, along with proliferation and migration of smooth muscle cells, may indicate angiogenesis pathways. In UM-3h only, proliferation and growth of muscle (z-score 3.9) were indicated, although the migration of smooth and skeletal muscle cells were shown in both datasets (z-score: 2.2). An inflammatory response was also indicated, specifically through inflammatory invasion, and also with enhanced immortalization, proliferation, and movement (including migration and elongation of cellular protrusions) of cells and tissues related to inflammation: lymphatic, bone marrow, blood, and hematopoietic progenitor cells. Pathway analysis suggested these various functions were driven by several signaling pathways that involved signaling mediators ERK1/2 and NFκB activity.

Analysis of the identified upstream pathways indicated abundant early ERK1/2 signaling (Tables 4.5a and 4.6a). Of regulators that met inclusion criteria,

nine are regulators within the ERK1/2 pathway, including MAPK3 (ERK1). Upstream, intracellular regulators of ERK1/2 activity RAF1, MAP2K1, MAP3K1, and TRAF6 were indicated to have increased activity. Further, activity of a downstream target of ERK1/2 and key transcription factor, CREB1, was enhanced (z-score: 5.3). Six out of seven secreted signaling molecules indicated through these analyses to have enhanced activity are extracellular regulators that activate the ERK pathway, including EGF and inflammatory cytokines IL5, IL1 β , and TNF. Among the top canonical pathways indicated to have enhanced activity were the ERK/MAPK pathway itself as well as extracellular stimulators of ERK (including IL6, CD40, HGF, and integrins), downstream products (IL8), and related inflammatory or proliferative pathways (p38 MAPK, PI3K/AKT) (Tables 4.5b and 4.6b). Taken together, these strongly indicate enhanced ERK activity and that ERK promoted pro-inflammatory signaling prior to and at 3h post-ECC. ERK and several interrelated pathways are also associated with pain or hyperalgesia, including the integrins and IL6. Furthermore, these analyses indicated that key 3h regulators that coordinate ERK activity (including ERK, MAP3K1, and RAF1) suppressed differentiation (muscle cells), and enhanced viability, proliferation, and migration (various cell types).

These analyses also indicated a signaling role (upstream and at 3h as a key regulator) for the canonical (p65) NF κ B pathway (activated). This pathway, critical to pro-inflammatory signaling, promotes transcription involved in proliferation, in part by interacts with ERK and CREB1—both of which were activated. Several extracellular regulators of NF κ B activity, including IL1 β , were also indicated to have

greater activity. Intracellular regulators that enhance NFκB activity were also activated, including MAP2K1 and TRAF6. Although the NFκB canonical pathway itself did not meet inclusion criteria, other canonical pathways strongly associated with NFκB activity (primarily to promote its activity) were indicated such as ERK, IL8, p38 MAPK, and CD40. Key regulators identified at 3h that directly relate to or are involved in activating the NFκB pathway included the NFκB itself, RelA, NFKBIB, and AREG. These regulators were identified in multiple pathways or functions and are also related to ERK activity.

Enhanced activity of other inflammatory pathways at 3h post-ECC was identified, although not as strongly as ERK and NFκB, including upstream regulation via the monocyte chemotaxis ligand CXCL12 and receptor CXCR4, JNK, and p38 MAPK. Beyond the pathways discussed above, no other inflammatory canonical pathways were identified to have increased or decreased activity at 3h. However, analysis indicated that activity of other inflammatory regulators was enhanced at 3h, many involved in ERK and NFκB signaling either up or downstream, including JAK2, CXCL12/CXCR4, IL2, IL2RB, IL1, CYR61, and F2RL. Downstream targets of these and the aforementioned ERK/NFκB-related regulators were upregulated and are involved in cell cycle progression and proliferation (e.g., FOS, CDKN1B, EGFR), extracellular matrix and connective tissue development (e.g., CTGF, CD44), and, most prevalently, inflammation (e.g., CYR61, CXCL1, IL1β, IL6, NFKBIA, NGF). The most dramatically induced genes in each dataset included: NR4A3 (UM-3h: 30.2-fold; MM-3h: 3.2-fold; interacts with CREB); CYR61 (29.6-fold; 7.6-fold); and MAP3K8 (13.8-fold; 11.0-fold; interacts with/activates JNK, NFκB, and ERK).

Shared Signaling at Early (3h) and Late (48h) Time Points Post-ECC

Unless otherwise indicated, z-scores presented below are the average of the two 3h datasets followed by the z-score at 48h (UM-3h and MM-3h/MM-48h).

Suppressed activity at 3h and 48h of the two alternative Wnt signaling canonical pathways, Wnt/Ca⁺⁺ (intracellular calcium signaling) and PCP (cell polarity), was inferred (Table 4.5). Analysis suggested that growth hormone and PPAR signaling were also suppressed, each of which may indicate attenuated JAK/STAT signaling—however, changes to that pathway did not reach statistical significance.

Enhanced upstream signaling in all three datasets may suggest anti-inflammatory signaling and continued movement toward muscle differentiation at or beyond 48h via MYOD1 (differentiation) and anti-inflammatory pathways including IL13, TGF β , IL4, and IL5. Yet the process of secondary damage and recovery is a continuum, and upstream signaling linked to early processes were also suggested via KRAS (proliferation/migration) and EIF4E (proliferation), CD38 (pro-inflammatory leukocyte migration), and NFE2L2 (oxidative stress). Identified functions neither supported nor refuted these data, although enhanced canonical pathway activity did lend support, including growth factors HGF and PDGF; acute phase response signaling; PI3K/AKT; and inflammatory signaling pathways IL6 and p38 MAPK, which can enhance pro- or anti-inflammatory signaling.

Contrasting Signaling at Early (3h) and Late (48h) Time Points Post-ECC

No canonical pathways or regulators that met inclusion criteria were indicated to have enhanced signaling at 48h that was suppressed or unchanged at 3h. Identified functions with this contrasting activity suggested greater import and metabolism of protein; augmented tissue development via increased mass of adipose and connective tissue; and infectious disease via viral release. The latter function may actually indicate the ejection of phagocytosed debris from inflammatory cells.

Only a handful of pathways and functions were indicated as enhanced at 3h and suppressed at 48h. Upstream regulators identified with this activity pattern are involved in angiogenesis (VEGFA) and inflammatory regulation: FN1, PRL, CD24, and ESRRA (Table 4.6a). FN1 and PRL are generally considered pro-inflammatory and PRL stimulates NF κ B activity. CD24 and ESRRA are considered anti-inflammatory through their roles in suppressing NF κ B (CD24) and PPAR (ESRRA) signaling. PPARG, a regulator with similar activity patterns, was suppressed at 48h, although PPARG canonical pathways were attenuated in all three datasets. Strictly oppositional signaling of canonical pathways only met inclusion criteria for cardiac hypertrophy signaling and the canonical Wnt/ β -catenin pathway (Table 4.6b). Two canonical pathways with little/no change at 3h and suppression at 48h did, however, meet inclusion criteria: androgen signaling (stimulates NF κ B) and ultraviolet (UVA)-induced MAPK signaling. Based on these and the remaining Canonical Pathways, early growth and pro-inflammatory signaling is followed by later suppression of each processes.

The majority of signaling differences were due to enhanced 3h signaling and little or no corresponding changes were observed at 48h, including the previously discussed CD40, CDK5, ERK/MAPK, IL8, and integrin signaling pathways (Table 4.6). These pathways have also been previously associated with hyperalgesia, and suggest several means through which: 1) there is a shift from primarily pro- (3h) to anti-inflammatory signaling (48h); and 2) hyperalgesia may be stimulated and resolve through the enhancement and subsequent suppression of various pathways. Important mediators of hyperalgesia and downstream targets at 3h but not 48h included a number of interleukins and NGF (see Early Signaling, above).

Together these data implicated ERK and NF κ B signaling as important contributing factors during the secondary damage phase after ECC. However, other pathways, such as AKT1, and functions were also implicated. Therefore, a smaller group of genes was tested using the more directed, custom-designed PCR arrays, qRT-PCR, and the canonical NF κ B pathway activity at 48h; these tests were used as confirmation to develop a more specific model of post-exercise responses.

Validation Studies

PCR Arrays

PCR arrays indicated significant upregulation of two genes in the exercised leg at 48h post-exercise as compared to baseline (Table 4.7, Supplemental). Of note, AKT1 mRNA (V-Akt Murine Thymoma Viral Oncogene Homolog 1, also known as Protein Kinase B or PKB), was upregulated 1.51 ± 0.07 -fold ($p < 0.002$). AKT1 has roles in cell cycle regulation (inhibiting proliferation) and signaling to promote

myogenesis. MAP2K1 was upregulated 2.48 ± 0.36 -fold ($p < 0.002$) post-exercise, is involved in proliferation, differentiation, and inflammation and it influences downstream signaling almost exclusively through the ERK and NF κ B pathways.

qRT-PCR

qRT-PCR was used to validate findings from the transcriptomic analyses that indicated potential involvement of NGF and of the AKT1, NF κ B, and ERK pathways in the shift from the early to late phases of secondary damage. Samples from UM-3h and VAL-48h were used to test three genes: TRAF6, NFKBIA, and NGF. TRAF6 regulates NF κ B activity, stimulates activation of JNK, p38 MAPK, AKT1, and, less commonly, ERK1/2 activity (152). NFKBIA is a negative regulator of the NF κ B p65 pathway, and lies downstream of TRAF6. NGF can stimulate activation of the NF κ B p65 pathway when bound to the low-affinity receptor, p75^{NTR}, and the ERK5 pathway when bound to the tyrosine receptor kinase A (TrkA) in endosomal transportation, and sustained activity of the ERK1/2 pathway when bound to TrkA at the cell surface. ERK1/2 and NF κ B can also enhance the expression of NGF. Changes to TRAF6 expression at 48h could indicate changes to the aforementioned pathways, while altered NFKBIA more specifically affects NF κ B (p65). Altered NGF could signal changes to downstream pathways NF κ B or ERK. Due to sample availability, six subjects were tested from UM-3h (VAL-3h), and five from VAL-48h. For analysis of NFKBIA, an outlier was removed from UM-3h. Each subset remained statistically similar to the total cohort for key demographic differences and strength loss.

TRAF6 was not differentially expressed either between time points or in response to exercise at either 3h or 48h (fold changes of 1.34 ± 0.24 and 1.15 ± 0.11 , respectively). Expression of NFKBIA was significantly altered with exercise ($p < 0.01$), with a decrease at 3h (0.56 ± 0.05 -fold) and return to pre-exercise levels by 48h (1.15 ± 0.23 -fold). The overall expression 48h post-exercise was greater than at 3h ($p < 0.05$), but there was no significant difference in upregulation between time points. For NGF, there was a significant upregulation after exercise ($p < 0.01$) that was greater at 3h (7.54 ± 2.28 -fold) than at 48h (1.94 ± 0.63 -fold, $p < 0.05$), with no overall effect of time. These findings are depicted in Figure 4.2.

NFκB Activity

The microarray, PCR array, and qRT-PCR data pointed to a suppression of NFκB p65 pathway activation at the 48h time point. I compared NFκB DNA-binding activity at 48h to previously measured NFκB p65 pathway activation at 3h post-exercise. Hyldahl et al. (137) reported a $230.5 \pm 51.2\%$ increase in NFκB activity at 3h after ECC (Figure 4.3). This upregulation was localized primarily (95% signal) to non-muscle cells. In contrast, at 48h after exercise NFκB activity was significantly suppressed ($142 \pm 10.8\%$ of the control leg, $p < 0.05$), constituting an approximate 315% difference in activity between the enhanced activation early and the later decrease. To test the relationship between NFκB and DOMS, early NFκB percent change and peak soreness were tested using correlations. Soreness was not tested in VAL-48h, so later NFκB activity could not be tested against soreness at this time. There was a moderate ($r = 0.55$) positive correlation between NFκB activation at 3h

and later peak soreness, which was significant ($p < 0.05$). These data suggest that NF κ B activity does influence DOMS, although the specific mechanisms through which this occurs are as of yet unknown.

Discussion

In this study, I tested the hypotheses that: 1) at 3h post-ECC, gene expression downstream of pro-inflammatory signaling such as IL1, IL6, and would increase, which could potentially stimulate NF κ B and related pathways to promote additional pro-inflammatory signaling; 2) at 48h post-ECC, gene expression would favor greater anti- and reduced pro-inflammatory signaling, for example increased gene expression downstream of anti-inflammatory proteins and cytokines such as IL4 and IL13; and 3) expression of genes related to hyperalgesia and/or DOMS would: a) increase at 3h; and b) decrease but remain elevated above baseline/control at 48h post-ECC. These data were then used to develop a model of the possible inflammatory signaling pathways that could mediate and heightened DOMS observed in smokers.

My first hypothesis, that early responses after ECC would favor pro-inflammatory signaling including NF κ B and related pathways, was supported. At 3h, canonical signaling pathways that stimulate pro-inflammatory activity and downstream signaling had increased z-scores—indicating greater likelihood of activation. These pathways, including IL-8, the integrins, and CD40, have all been previously linked to ERK1/2 activity. NF κ B activity was also indicated, primarily as a key regulator of numerous functions and pathways, although primarily in UM-3h.

Furthermore, NGF, which can mediate hyperalgesia, was identified as a target molecule at this time, was upregulated in both 3h datasets, and is also connected to, in some cases integrally, each of the pro-inflammatory signaling pathways previously discussed.

Including multiple protocols enhanced the study: determining shared responses at the 3h time point, independent of protocol differences, provides a better understanding of fundamental mechanisms early after muscle damage. For example, fibroblast growth factor (FGF) signaling was slightly suppressed in UM-3h (z-score: -0.39) yet enhanced in MM-3h (z-score: 2.33), suggesting it is not an inherent signaling pathway to generalized ECC response. Generally, the support for evidence of pro-inflammatory signaling (indicated through z-scores and significance) was stronger in UM-3h than MM-3h, for example IL-8 signaling (z-scores: UM-3h, 1.94; MM-3h, 1.15) and NF κ B (as an upstream regulator; UM-3h, 6.20; MM-3h 4.39). This may have been due to differences in study protocol—the slower speed of each eccentric contraction in UM-3h (and both VAL groups), as compared to MM, meant a more prolonged time under tension creating more muscle stress and, possibly leading to greater subsequent damage and inflammatory responses. Altogether these data suggested that the degree of pro-inflammatory response is dependent upon the level of muscle stress, and this may explain the discrepancy in inflammatory signaling (some with, others without) between reports of post-ECC responses (200, 247, 267, 270).

Results from transcriptome analyses at the later time point (48h post-ECC) were less clear than those at 3h post-ECC. I had originally expected signaling at 3h to

promote pro-inflammatory pathways and at 48h to directly oppose these pathways through enhanced anti-inflammatory signaling. Instead, pro-inflammatory pathways enhanced at 3h were simply no longer differentially expressed at 48h. The primary exception to this was MAPK signaling (UVA-induced), which was suppressed at 48h, yet had not been enhanced early. However, given that the earlier, induced pathways were related to ERK and NF κ B signaling, and that these pathways appeared to have ceased their supranormal signaling by 48h, suggests a general suppression of ERK- and NF κ B-related signaling. The disparity between the results found and those expected may have stemmed from the exercise protocols. The MM study protocol (faster rate of contraction) was more similar to a fatiguing protocol while the UM and VAL study protocols (greater time under tension) were developed to elicit a higher level of muscle stress and damage. Molecular responses at 48h from MM support greater effects of fatigue rather than high levels of damage. While this did provide a general understanding of processes happening at 48h, the gene expression differences at 3h indicated that other processes may occur at 48h with greater perturbation. Thus, to more fully understand responses to damage, further investigation at 48h was required using the protocol from UM/VAL.

To filter candidate pathways from transcriptome analysis and extend these to the 48h time point using the same exercise protocol as the UM-3h dataset, PCR arrays were used to test genes related to pathways and functions indicated through transcriptome analysis. The immediate upstream regulator of ERK1/2, MAP2K1, was also upregulated at 48h. To date, a single published study (335) has shown increased activity of the ERK pathway beyond the early muscle damage response

(ERK). ERK activity can be transient or sustained, each of which initiates different downstream signaling (1, 296). Therefore, the role of ERK at 48h after ECC may differ from its early activity. qRT-PCR was then used in an attempt to clarify the signaling at 48h and provide additional information regarding hyperalgesia-related signaling.

To further clarify ERK and NFκB signaling at 48h post-ECC, I performed qPCR analyses and tested NFκB DNA-binding activity. Suppressed expression of NFKBIA at 3h, that was not different from baseline (control) at 48h from baseline, support the transcriptome analysis results indicating suppressed NFκB activity at the late time point. NFκB activity was increased at 3h but suppressed at 48h compared to controls. This is novel finding in humans after ECC. Research has previously shown canonical NFκB pathway activation changes during muscle proliferation and differentiation (13), in which this pathway is activated early and stimulates both proliferation and pro-inflammatory signaling. Then, approximately 48h post-ECC, activity is attenuated, pro-inflammatory signaling begins to resolve, and differentiation ensues. It is therefore possible that the resolution of NFκB activity may be an important, if not critical, step in the conclusion of the early pro-inflammatory phase and forward progress toward healing and regeneration. In addition to its role in proliferation, NFκB may be a hyperalgesic-mediator (reviewed in Haddad (111)). In this study, I found that early NFκB activity was moderately correlated with peak DOMS expression, and therefore may be involved in DOMS signaling.

Because NGF binding can drive ERK and NFκB signaling, and those pathways can in turn stimulate NGF expression, I extended the findings related to hyperalgesia by testing NGF expression. As anticipated, NGF expression was elevated at 3h and, while somewhat attenuated in degree, remained so at 48h. These data support previous research regarding NGF and ECC (233, 234) and extend such research to humans at multiple time points after ECC. The mechanisms underlying the resolution of DOMS are poorly understood, yet these data suggest that as NFκB activity wanes, so do NGF expression and hyperalgesia.

Based on the findings of this study, I have developed a model to explain the signaling shifts between early and late phases of inflammation that could influence DOMS. In this model (Figures 4.4 and 4.5), early extracellular signaling, including pro-inflammatory cytokines and NGF, stimulate activity of the NFκB and ERK pathways, resulting in the production of additional pro-inflammatory signaling molecules. Later, as extracellular pro-inflammatory (and NGF) signaling wanes, so does NFκB activity, resulting in further de-activation of pro-inflammatory signaling and hyperalgesia. If ERK activity is enhanced and, if so, whether that activity is transient or sustained, requires additional study in future. Furthermore, the specific ways in which NGF exerts its downstream activity (e.g., to which receptor it binds and therefore which downstream pathways it activates) after ECC are currently unknown. NGF binds to two different receptors and can instigate disparate downstream signaling and responses based on that binding. Therefore, a greater understanding of NGF signaling after ECC is warranted to further our knowledge of its role in DOMS.

These analyses were performed to develop a working model of mechanisms underlying muscle damage and repair responses, specifically DOMS, and to develop a model to use for future evaluation of heightened DOMS in cigarette smokers after ECC. In a previous study (Dissertation Study 1, Chapter 3) I found that smokers have greater peak DOMS. Smoking has also been previously associated with changes in NGF expression and activity (primarily enhanced) of both ERK and NF κ B. Therefore, in the final chapter of my dissertation, I describe the results of a study designed to test this model in chronic cigarette smokers who engaged in ECC in order to elucidate the cause of greater DOMS and explore possible muscular consequences of smoking.

Tables and Figures

Table 4.1: Simplified Timeline of Study Visits.

Studies were performed at multiple sites and muscle samples collected early (3h) or late (48h) after eccentric exercise (ECC). UM-3h, University of Massachusetts Amherst-3h; VAL-3h, validation study at University of Massachusetts Amherst-3h; MM, McMaster University, samples at both 3 and 48h; VAL-48h, validation study at University of Massachusetts Amherst-48h. CK, creatine kinase.

Time	Measure	UM-3h VAL-3h	MM	VAL-48h
Pre-ECC	CK	X	X	X
	Strength	X		
	Biopsy (control)		X	
	Soreness	X		
Exercise	Pre-exercise strength	X		X
	Biopsy (control)		X	
	Exercise	X	X	X
	Post-exercise strength	X		X
3h post-ECC	Biopsy	X	X	
	CK		X	
1d post-ECC	Soreness	X		
	CK	X		X
	Strength	X		X
2d post-ECC	Soreness	X		
	CK	X	X	
	Strength	X		
	Biopsy		X	X

Table 4.2. Biological Pathway Analysis Criteria.

Criteria used for transcriptome analyses using Ingenuity Pathway Analysis.

See Supplemental page 1

Table 4.3: qRT-PCR Primers.

NGF, nerve growth factor; NFKBIA, Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; TRAF6, TNF receptor-associated factor 6; B2M, Beta-2-microglobulin.

Target	Primer	Reference
NGF	Fwd: 5'- CAACAGGACTCACAGGAGCA -3'	(2)
	Rev: 5'- ACCTCTCCCAACACCATCAC -3'	
NFKBIA	Fwd: 5'-TGG CCT TCC TCA ACT TCC AGA ACA-3'	(137)
	Rev: 5'-CTC AGC AAT TTC TGG CTG GTT GGT -3'	
TRAF6	Fwd: 5'-TGATAGTGTGGGTGGAAGT-3'	(282)
	Rev: 5'-CTCCTTGGACAATCCTTCAG-3'	
B2M	Fwd: 5'-TGTCTGGGTTTCATCCATCCGACA-3'	(137)
	Rev: 5'-TCACACGGCAGGCATACTCATCTT-3'	

Table 4.4: Subject Characteristics.

Data are presented as mean±SEM. Body mass index (BMI) data are unavailable for the MM study. UM-3h, University of Massachusetts Amherst-3h; VAL-3h, validation study at University of Massachusetts Amherst-3h; MM, McMaster University; VAL-48h, validation study at University of Massachusetts Amherst-48h.

	N	Age (y)	Height (cm)	Weight (kg)	BMI
UM-3h	35	20.9±0.5	178.9±1.2	80.6±2.9	25.2±0.8
VAL-3h	6	19.3±0.6	174.8±3.2	78.5±5.3	26.1±2.0
MM	9	21±2.4	181.6±5.6	73.4±1.5	-
VAL-48h	9	22±0.8	180.7±2.0	82.2±2.8	25.9±0.3

Table 4.5: Early and Late Time Points: Shared Transcriptome Responses.

Ingenuity Pathway Analysis of microarray data at 3 and 48h post-exercise regarding (a) Upstream Regulators and (b) Canonical Pathways. Data are presented as z-scores, darker color greater up- or down-regulation: green is decreased activity, red is increased activity. Gene names and symbols from the National Institute of Health (NIH) National Center of Biotechnology (NCBI) database, June 2015. * $p < 0.05$. UM, University of Massachusetts; MM, McMaster University.

Upstream regulator	Gene name	UM-3h	MM-3h	MM-48h
		z-score		
CD3	CD3g molecule, gamma (CD3-TCR complex)	-2.3 *	-2.6 *	-1.4 *
KMT2A	Lysine (K)-specific methyltransferase 2A	-0.9 *	-1.2 *	-2.0
SYVN1	Synovial apoptosis inhibitor 1, synoviolin	2.6 *	0.9 *	0.9 *
E2F1	E2F transcription factor 1	1.0 *	1.2 *	1.7 *
MYOD1	Myogenic differentiation 1	1.0 *	1.5 *	1.9
IL13	Interleukin-13	1.4 *	1.6 *	1.9
EIF4E	Eukaryotic translation initiation factor 4E	2.5 *	2.0	1.9 *
KRAS	Kristen rat sarcoma viral oncogene homolog	1.7 *	2.2 *	1.5 *
CD38	CD38 molecule	2.8 *	2.4 *	1.3 *
Tgf beta	Transforming growth factor	3.3 *	2.5 *	1.8
NFE2L2	Nuclear factor erythroid 2-like 2	3.8 *	2.7 *	3.0 *
IL4	Interleukin-4	4.0 *	2.7 *	2.1
IL5	Interleukin-5	6.0 *	3.9 *	2.7
TGFB1	Transforming growth factor B	8.1 *	4.6 *	2.1

Canonical pathway	UM-3h	MM-3h	MM-48h
	z-score		
Wnt/Ca+ pathway	-1.3 *	-1.2 *	-2.3 *
PCP pathway	-1.6 *	-1.7 *	-2.2
Growth hormone signaling	-1.2	-0.6 *	-1.4
PPAR signaling	-2.2 *	-0.3 *	-1.3
IL-6 signaling	2.5 *	2.4 *	1.2
PI3K/AKT signaling	2.2 *	0.8 *	1.2 *
HGF signaling	0.8 *	1.7	1.3
PDGF signaling	1.1 *	1.1 *	1.5 *
p38 MAPK signaling	2.3 *	1.5 *	1.6
Acute phase response signaling	2.4 *	0.8	1.7

Table 4.6: Early and Late Time Points: Contrasting Transcriptome Responses.

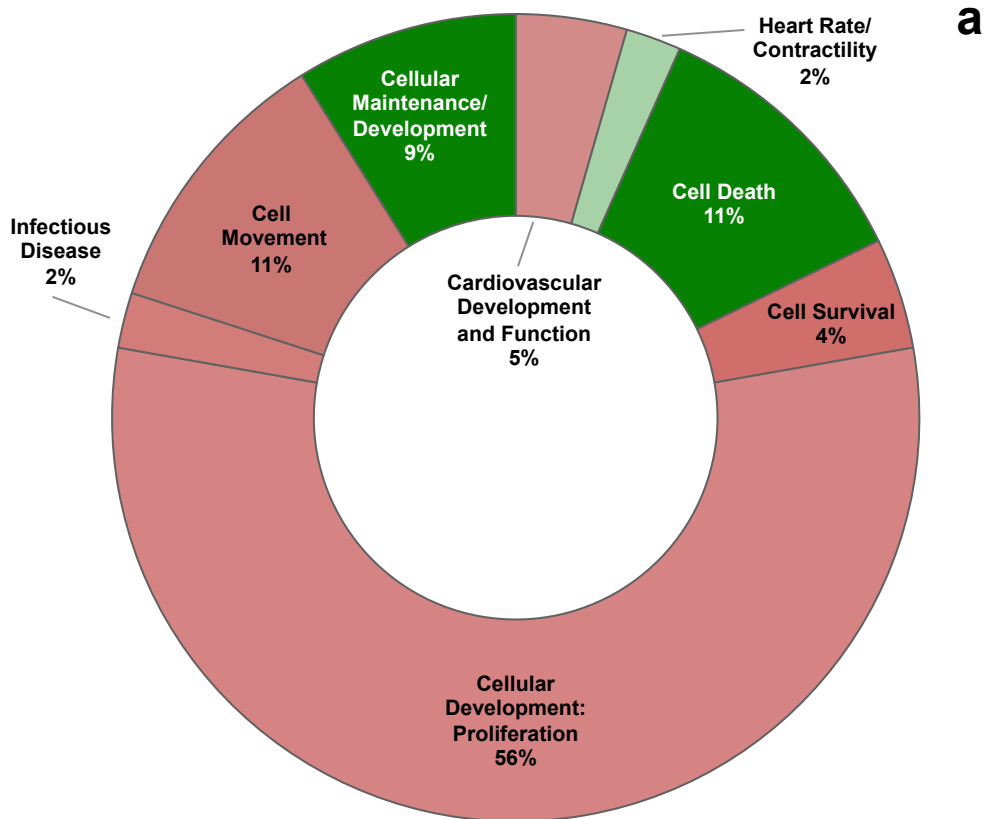
Ingenuity Pathway Analysis of microarray data at 3 and 48h post-exercise regarding (a) Upstream Regulators and (b) Canonical Pathways. Data are presented as z-scores, darker color indicates more up- or down-regulation: green is decreased activity, red is increased activity. Gene names and symbols from the National Institute of Health (NIH) National Center of Biotechnology (NCBI) database, June 2015. *p<0.05. UM, University of Massachusetts; MM, McMaster University.

Upstream regulators	Gene name	UM-3h	MM-3h	MM-48h
		z-score		
FN1	Fibronectin 1	2.9 *	2.8 *	-2.4 *
VEGFA	Vascular endothelial growth factor A	4.2 *	3.0 *	-2.1
PRL	Prolactin	3.0 *	1.8 *	-1.9
CD24	CD24 molecule	1.3 *	2.3 *	-1.9 *
ESRRA	Estrogen-related receptor alpha	1.9	1.1 *	-1.5 *

Canonical pathway	UM-3h	MM-3h	MM-48h
	z-score		
Agrin interactions at neuromuscular junction	2.0 *	2.5 *	-0.8
Androgen signaling	0.3		-1.3 *
Cardiac hypertrophy signaling	2.0	0.8 *	-1.6 *
CD40 signaling	1.4 *	2.1	0.5
CDK5 signaling	2.1	1.8 *	-0.6
ERK/MAPK signaling	1.4 *	0.7 *	-0.5
ERK5 signaling	1.4 *	2.3 *	0
HMGB1 signaling	1.6 *	0.8	-0.3
IL-8 signaling	1.9 *	1.2	0
Integrin signaling	2.7	3.1 *	-0.4 *
PI3K signaling in B lymphocytes	1.5	1.3 *	0 *
UVA-induced MAPK signaling	-0.5	0.3	-2.5 *
Wnt/ β -catenin signaling	-1.3 *	-1.5 *	0.7

Figure 4.1: Early (3h) and Late (48h) Post-ECC: Transcriptome Functions.

Overarching functions identified by Ingenuity Pathways Analysis from (a) early (3h encompasses UM-3h and MM-3h) and (b) late (48h, MM only) transcriptome data. Darker color indicates greater up- or down-regulation: green is decreased activity, red is increased activity. The number of specific functions within each category are presented as a percent of the total identified specific functions at that time point. UM, University of Massachusetts; MM, McMaster University.



b

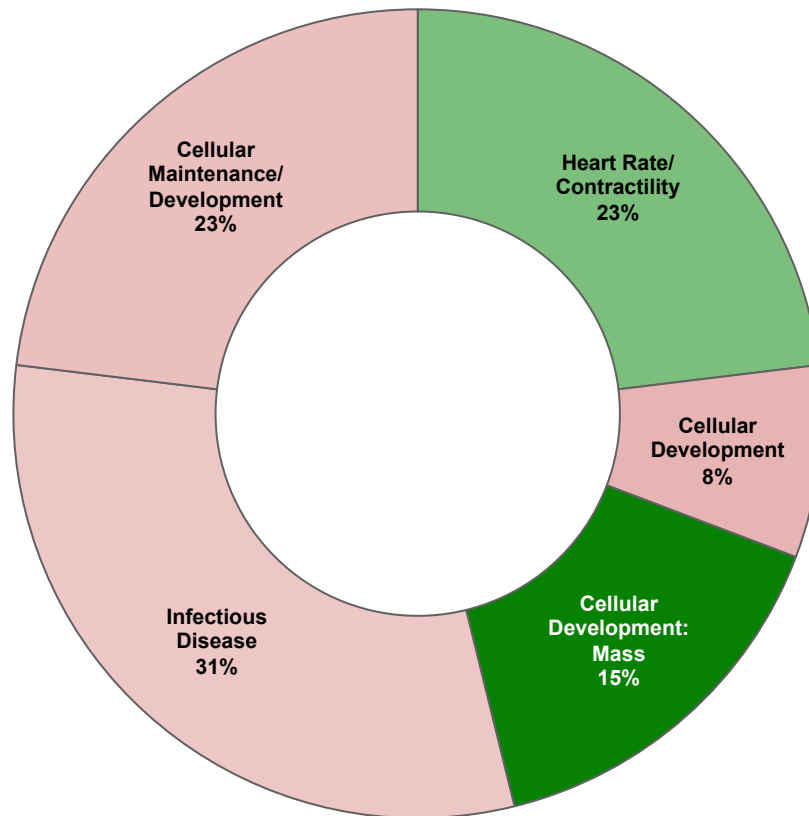


Table 4.7: PCR Array Analysis.

The array was comprised of 44 genes of interest normalized to two housekeeping genes. Gene names and symbols from the National Institute of Health (NIH) National Center of Biotechnology (NCBI) database, June 2015. Functions from NCBI, UniProt, Genecards, and published literature. Data are presented as fold change (exercise vs control leg, mean \pm SEM) and were analyzed as $\Delta\Delta C_t$ using repeated measures ANOVA, with significance based on false discovery rate: * $p < 0.002$, effect of exercise. ANOVA, analysis of variance.

See Supplemental page 2

Figure 4.2: qRT-PCR Validation.

Post-ECC gene expression. Early (3h) , late (48h) . Data are presented as mean \pm SEM. * $p < 0.05$ effect of exercise; § $p < 0.05$ interaction (group*exercise). NGF, nerve growth factor; NFKBIA, nuclear factor kappa B inhibitor-alpha; TRAF6, TNF-associated factor 6.

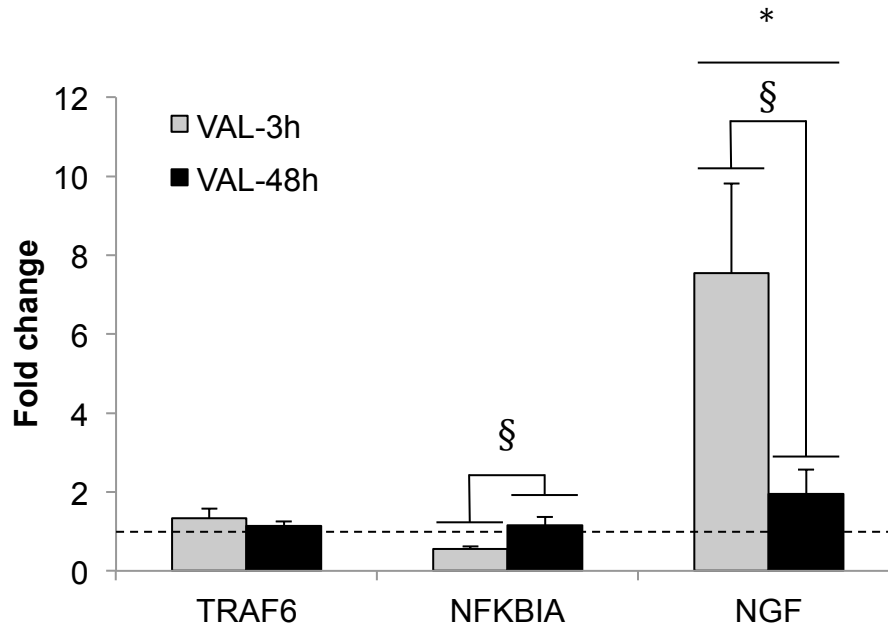


Figure 4.3: NFκB Activity.

Post-ECC NFκB (p65) DNA-binding activity. Early (3h) , late (48h) . Data are presented as mean \pm SEM. * $p < 0.05$ effect of exercise. NFκB, nuclear factor kappa beta; p65 (REL-A), v-rel avian reticuloendotheliosis viral oncogene homolog A.

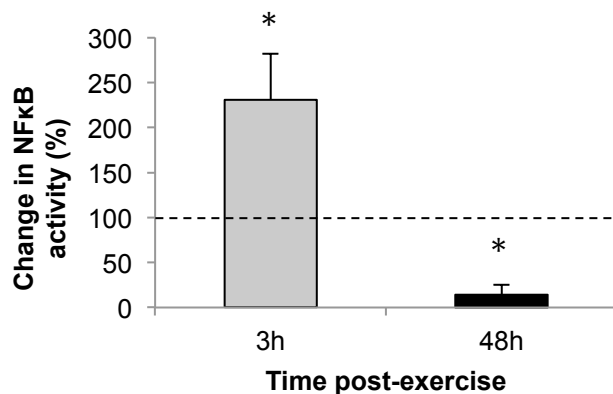
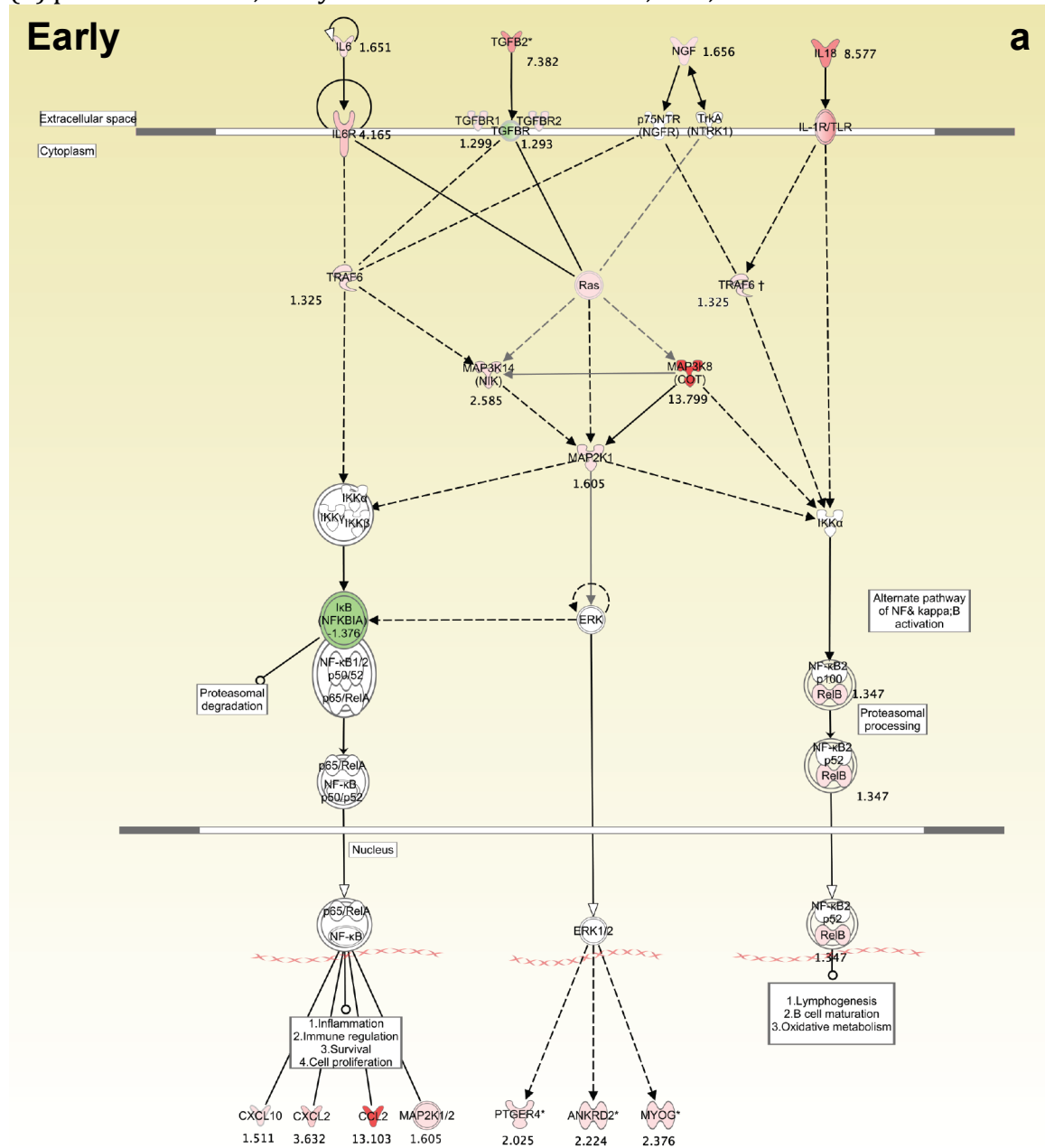
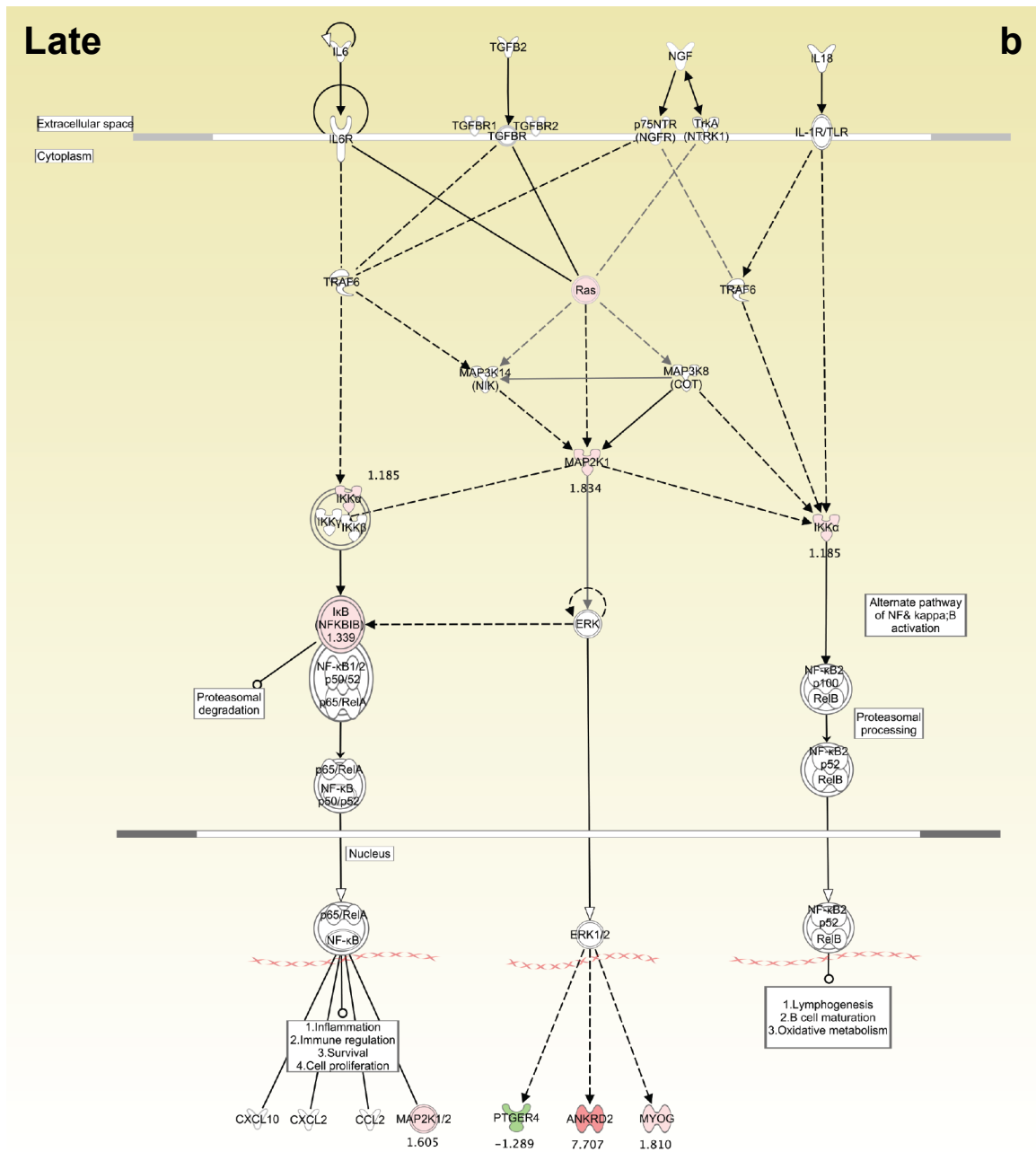


Figure 4.4: Proposed Model of Inflammatory Events at 3h and 48h Post-ECC.
Proposed pathway regulation affecting inflammation and DOMS early (a) and late (b) post-ECC. DOMS, delayed onset muscle soreness; ECC, eccentric exercise.





CHAPTER V
MOLECULAR EFFECTS OF SMOKING ON SKELETAL MUSCLE
48H AFTER ECCENTRIC EXERCISE

Introduction

The long-term negative consequences of cigarette smoking include an increased risk for musculoskeletal injury (5, 47, 48, 150, 157, 212, 286, 343, 363) and prolonged healing time (3, 82, 310, 317, 357). In a previous study investigating smoking and injury risk, I used an eccentric exercise model (ECC) of acute muscle injury that elicits transient muscle damage, including delayed hyperalgesia (delayed onset muscle soreness, or DOMS) and functional losses (reviewed in (135). In response to ECC, smokers experienced greater muscle fatigue, as indicated by 15% greater eccentric torque loss during and 10% greater loss of strength 5min after ECC, and 15% greater peak DOMS than non-smokers (Dissertation Study 1, Chapter 3). Since the mechanisms responsible for the link between smoking and muscle injury risk are poorly understood, I conducted a molecular analysis of muscle biopsies from smokers and non-smokers after ECC to better understand why smokers demonstrate higher fatigue and DOMS.

Muscle fatigue may be affected by a number of mechanisms (reviewed in Allen, Lamb, and Westerblad (4)) including fiber type distribution. A growing number of studies have shown muscle of smokers to favor a lower proportion of Type I (oxidative) fibers than in non-smokers (169, 290, 328). Type I muscle fibers are more fatigue resistant than Type II fibers, and therefore a fiber-type shift away

from Type I fibers could predispose smokers to greater fatigue during exercise. Recent studies suggest that smoke exposure may influence muscle fiber type through the suppression of peroxisome proliferator-activated receptor-gamma (PPAR- γ) coactivator-1 (PGC-1) (162, 328), a family of proteins that promote oxidative (Type I) muscle fiber development. The non-canonical NF κ B (REL-B, v-rel avian reticuloendotheliosis viral oncogene homolog B) pathway regulates PGC1, promotes oxidative metabolism, and is enhanced during muscle differentiation (13). Smoking affects REL-B activity (375) and may be a mechanism through which fiber type shifts enhance muscular fatigue in smokers.

DOMS is driven in part by inflammatory signaling after ECC that is critical for muscle repair. Early pro-inflammatory activity (0 to ~2d post-exercise), exacerbates mechanical damage, while later anti-inflammatory activity (>2d post-exercise) quenches inflammation and stimulates healing and recovery. The resolution of pro-inflammatory activity is critical to the recovery process, as delayed resolution can lead to “secondary” muscle damage to the surrounding healthy tissue and stimulate DOMS (reviewed in Hyldahl and Hubal (135)).

Non-ECC models of pain have identified several early pro-inflammatory mediators in hyperalgesia signaling through the sensitization of nociceptors. In the well-characterized cyclooxygenase (COX) pathway, COX stimulates the synthesis of prostaglandins, which induce hyperalgesia (87, 201, 233). Bradykinin (B2) stimulates hyperalgesia via ion channel activation and can stimulate the production and release of prostaglandins, which can, in turn, potentiates the nerve-sensitizing effects of B2 (234). Nerve growth factor (NGF), which also mediates prostaglandins

and B2, sensitizes nociceptors directly and indirectly by stimulating neutrophils and mast cells to release factors such as tumor necrosis factor- α (TNF- α) (reviewed in (224)). Recent evidence indicates that NGF is an important mediator of DOMS (234).

My previous transcriptome analyses (Dissertation Study 2, Chapter 4) suggested differential activation of the extracellular regulated kinase (ERK) and canonical (p65: REL-A, v-rel avian reticuloendotheliosis viral oncogene homolog A) nuclear factor kappa B (NF κ B) pathway between 3h (up vs control) and 48h (down vs control) after ECC in humans. Both pathways can stimulate hyperalgesia in non-ECC pain models by regulating the synthesis and release of other hyperalgesic and pro-inflammatory factors, such as the COX2 pathway and TNF- α , yet their effects on DOMS have yet to be determined. ERK can promote pro- or anti-inflammatory signaling and regulate inflammatory cell survival or apoptosis via its activity in the nucleus (including inflammatory gene expression) and cytoplasm (where it can regulate p65 activity). After ECC, ERK is activated early, yet later activation may also occur (335). Other hyperalgesic factors were also differentially expressed between these two time points. This included NGF, which stimulates ERK and p65 activity and (separately and together) can stimulate the production and release of NGF (51, 85, 172, 174, 238, 261). NGF was enhanced at 48h, but to a greater extent at 3h. Together, these pathways may promote DOMS, which resolves as their activity subsides.

Smoking is associated with dysregulated inflammation, including NGF expression (elevated with smoke components (238, 368)) and ERK and p65

activation (6, 238, 368, 385)—all of which are associated with hyperalgesia. Chronic cigarette smokers report greater pain after traumatic injury or surgery (103, 287), higher incidences of chronic pain (including musculoskeletal pain (386), and they require larger doses of analgesics to manage pain (68, 288)). Differences in nerve-sensitizing pathways may explain heightened DOMS in my previous research and shed light on enhanced muscle injury risk and prolonged recovery.

The aims of the present study were to determine if: 1) REL-B activity and fiber type distribution differed between muscle of smokers and non-smokers; and 2) NFκB and ERK activity, along with NGF protein levels, were altered in smokers. These mechanisms may explain greater muscle fatigue and DOMS in smokers, respectively (Dissertation Study 1, Chapter 3). I hypothesized that: 1) smokers would have a greater proportion of Type II fibers and suppressed REL-B activity at 48h post-ECC compared to non-smokers; 2) after ECC the activity of the canonical NFκB pathway would be suppressed in non-smokers but not in smokers; 3) nuclear activity of the ERK pathway would increase for non-smokers while activity would be concentrated in the cytoplasm for smokers; and 4) NGF levels and receptor co-localization would be more pronounced in smokers versus non-smokers.

Methods

This study was comprised of two parts: 1) an *in silico* transcriptomic profile analysis; and 2) an experimental analysis validating key pathways identified through prior research and the *in silico* analyses. The *in silico* analysis was used to determine which damage-related genes may also be altered by smoking and/or

pain. Then, I tested the combined effects of exercise and cigarette smoking on signaling components of the identified pathways.

***In Silico* Expression Profile Analyses**

The National Institutes of Health Gene Expression Omnibus (GEO) profile database (<http://www.ncbi.nlm.nih.gov/geoprofiles>) was used to query the effects of: 1) muscle damage; 2) smoking; and 3) pain on 21 genes (Table 5.1, Supplemental), selected based on models developed from previous transcriptome analyses (Dissertation Study 2, Chapter 4). The primary goal was to identify which genes from the model may be involved with hyperalgesia signaling or affected by smoking. There are currently no studies in the GEO database that report on investigations of the effects of smoking on skeletal muscle, and datasets related to the effects of cigarette smoking, tobacco use, or exposure to chemicals associated with cigarette use on other tissues were queried. I chose 24 data sets for further analysis using the following search terms: 1) muscle damage/injury (search input: muscle, skeletal muscle, injury, damage, eccentric, exercise, dystrophy); 2) cigarette smoking/smoke exposure (cigarette, smoke, smoking, tobacco); and 3) models of pain/hyperalgesia (pain, hyperalgesia). The raw data were downloaded and then mRNA expression was probed using ANOVA testing for statistically significant differences: 1) after muscle damage; 2) with smoke exposure/smoking; or 3) with pain stimulation.

Validation Study

Subjects

Twenty healthy young men, ages 18-35, participated in this study. All subjects were generally sedentary (all regular activities below 5 METs) and had not performed resistance training with their legs or engaged in any heavy lifting/lowering of materials for the previous six months, verified through interviews and the Paffenbarger activity questionnaire (259). Throughout the course of the study, subjects refrained from: new physical activities; consumption of alcohol or dietary supplements; muscle treatments (including hot/cold or massage treatments) or use of any medications that might affect the study outcome, except acetaminophen after the biopsies.

The non-smokers (N=10) recruited for the study had never smoked regularly nor consumed any tobacco/nicotine products. The chronic cigarette smokers (N=10) had habitually smoked at least ½ pack (10 cigarettes) per day for five or more years, without the consumption of other tobacco products. The smokers were required to sustain their normal smoking habits, as well as consume one cigarette 10min before each visit to reduce variability that might occur as a result of time from last cigarette. Prior to participating, all subjects gave written informed consent as approved by the University of Massachusetts Amherst Institutional Review Board.

Experimental Design

A summary of study events is provided in Figure 5.1, with details below. All visits took place around mid-day, except the third and fourth visits, which occurred in the morning (see below for details). Subjects fasted overnight (no food or drink except water) in advance of the first, third, and fourth visits. At V1, subjects provided a medical history, filled out the Paffenbarger activity questionnaire, performed a familiarization session with the Biodex dynamometer for future strength testing and exercise, and had a baseline blood draw. Strength was tested using a Biodex dynamometer, specifically testing isometric maximal voluntary contractions (MVCs). Within 2-4d, subjects returned to the laboratory (V2) and had baseline strength measured, performed the exercise session, and had strength measures taken again at 5min post-exercise. At V3, which took place the morning after the exercise session at approximately 20h post-exercise, blood was drawn and strength again tested. At V4 (2d post-exercise), subjects came to the laboratory in the morning, consumed a standardized meal consisting of 400 kcals (approximately 55% carbohydrate, 30% fat, and 15% protein), and then remained resting the laboratory until the biopsy collection approximately 3-4h later. The last two visits took place at 4 and 9d post-exercise (2 and 7d post-biopsy) and consisted of strength testing and an examination of the biopsy site. The sutures were removed at the last scheduled visit, or one week following the biopsy to allow additional healing, by the study physician.

Blood Collection and Creatine Kinase Activity Analysis

Blood was collected under fasting conditions at the antecubital region by venipuncture using standard aseptic technique. For creatine kinase (CK) activity analysis, blood was drawn into vacutainers containing the anticoagulant EDTA. After resting at room temperature for 15min, the vacutainers were centrifuged for 10min at 1000 x g. The resultant supernatant (plasma) was shipped to a clinical laboratory for CK activity analysis using standard ELISA-based technique (Holyoke Hospital, Holyoke, MA).

Isometric Maximal Strength Testing

Functional testing was performed exclusively with the non-dominant leg. Isometric maximal voluntary contraction (MVC; measure of muscle strength) was tested to verify the efficacy of the exercise protocol in eliciting muscle dysfunction (pre-, 5min, and ~1d post-exercise) and to ensure proper recovery (4 and 9d post-exercise). While the primary focus of the study was the knee extensor muscle group, I also examined the flexor muscle group (changes in flexion strength were not anticipated). All strength measures and eccentric exercise were performed on a Biodex System 3 dynamometer (Biodex Medical Systems, Shirley, NY, USA) as described previously (6, 14). In brief, subjects were seated on the dynamometer and straps were placed across the upper leg (non-dominant only), hips, and chest to moderate the use of muscles not under investigation. The dynamometer arm pivot point was aligned with the lateral epicondyle of the femur. Before testing, the study investigator moved the subject's relaxed leg through passive range of motion. At full

extension of the knee, the dynamometer was set to 0° ; isometric ($0^{\circ}/\text{sec}$) and strength was measured at 70° of flexion. Subjects performed six total contractions against the immovable dynamometer level arm, alternating between extension (kicking) and flexion (pulling) for a total of three contractions each. Each contraction was 4sec in length and followed by 60sec of rest. The study investigator provided verbal encouragement to reinforce maximal effort. For each set, all repetitions were within 10% coefficient of variation.

Eccentric Exercise

The exercise session was comprised of 100 (10 sets of 10 repetitions) maximal eccentric contractions of the knee extensors on the Biodex dynamometer (6, 14). Subjects were seated as described above and the leg moved passively to 35° of flexion by the study investigator. Subjects then were instructed to kick with maximal effort throughout the exercise. Eccentric resistance was set at a moderate pace of $30^{\circ}/\text{sec}$, such that although the subject provided maximal effort, his knee was forced to move into flexion. At full flexion, the resistance would stop, and the subject's leg was returned passively by the study investigator to 35° of flexion—a 75° range of motion. Each contraction was approximately 4sec in length, with 10sec rest between repetitions. Each 10-repetition set was followed by a 1min rest period. Throughout the exercise session, subjects were verbally encouraged to exercise with maximal effort. Work done and eccentric torque were recorded for each contraction.

Muscle Biopsy

Biopsy procedures have been reported previously (6). Briefly, the study physician collected percutaneous needle biopsies from the vastus lateralis under local anesthetic (lidocaine) using a Bergström needle with suction. Each biopsy site yielded approximately 100-150mg of tissue. After fat and connective tissue were removed, the skeletal muscle was flash-frozen in liquid nitrogen and stored at -86°C until processed. Two biopsies were collected from each subject, first from the exercise leg and then immediately after from the control leg. The biopsy collection process began 48h after the exercise session and took approximately 15-20min for each leg. Subjects were permitted use of acetaminophen after the biopsy to manage pain associated with the biopsy procedure; all subjects ceased use of acetaminophen within 3d.

RNA Isolation

Total RNA was extracted using the RNeasy RNA isolation kit (Qiagen, Valencia, CA, USA). Samples were resuspended in TE buffer (pH 8.0) and the quality and quantity of RNA was tested in duplicate using spectrophotometry (Nanodrop, Wilmington, DE). Samples with a 260/280 ratio below 1.5 were subjected to an additional clean up step using the RNeasy kit. cDNA was then synthesized for qRT-PCR from 0.5ug of total RNA with a RT² First Strand cDNA synthesis kit (SABiosciences, a part of Qiagen) or for PCR arrays from 0.04ug total RNA with a RevertAid First Strand cDNA synthesis kit (Fermentas, Thermo-Fisher Scientific, Rockford IL, USA). All kits were used per manufacturer's instructions.

PCR Arrays

PCR arrays were used to quantify the mRNA expression levels of 44 genes (Table 5.1) related to pathway components of the muscle damage/regeneration response and to compare levels between smokers and non-smokers (SABiosciences, a part of Qiagen). The genes on this custom-designed array were chosen to investigate hypothesized differences between smokers and non-smokers based on previous literature regarding the effects of smoking and hyperalgesia, in addition to the microarray data explored in Dissertation Study 2 (Chapter 4). These genes encode for proteins with functions in six overarching categories: apoptosis, cell cycle, hyperalgesia, inflammation, muscle structure, myogenesis, nitric oxide signaling, and vascular health/angiogenesis (1-5, 7, 8, 11-13).

cDNA (1.5ng/uL final concentration) combined with RT² SYBR Green qPCR master mix was added to a microtiter plate containing the primer sequences (10uM concentration) for each gene of interest (quality assurance testing, including melting curve analyses, were performed by Qiagen). After 10min at 95°C to activate the polymerase, samples were amplified over 40 heating/cooling cycles of 95°C for 15sec followed by 1min at 60°C; PCR was performed on an MX3000p Real-Time PCR System (Stratagene, La Jolla, CA, USA). The average threshold cycle (C_t) for each gene of interest was then normalized to the average of two housekeeping genes that do not change in response to resistance exercise (9): β -actin and β -2-microglobulin (B2M), yielding ΔC_t for each leg. The ΔC_t for the exercise leg was then normalized to

that from the control leg, and the resultant $\Delta\Delta C_t$ were transformed into fold-change for visualization purposes.

qRT-PCR

To confirm and extend findings from the previous transcriptome work (Dissertation Study 2, Chapter 4), *in silico* data presented here, and the PCR arrays, I also performed semi-quantitative real-time PCR with primers for nerve growth factor (NGF), nuclear factor κ B inhibitor- α (NFKBIA), and TNF-associated factor-6 (TRAF6). The primer sequences with the number of subjects per group are presented in Table 5.2. Primer sequences were utilized at a concentration of 6 μ M. Quality of the primers was tested via RT-PCR and agarose gel electrophoresis. In each case, ethidium bromide staining was used to verify the existence of a single product that was the anticipated size. Due to limited sample availability, only samples from a sub-set of subjects were used for qRT-PCR analysis. This group did not differ from the entire cohort for age, height, weight, or BMI.

The relative abundance of the transcripts for the genes of interest was measured by qPCR analysis using SsoFast Evagreen master mix (Biorad, Hercules, CA). For each gene of interest, samples (cDNA final concentration of 25ng/ μ L) were run in triplicate in 96-well plates along with no template controls. A melting curve was generated following each reaction in order to insure that no primer dimers were detected. The triplicate C_t values were averaged and normalized to the housekeeping gene B2M, validated previously to maintain expression after eccentric exercise (9). Time and group comparisons were made using the $\Delta\Delta C_t$ method.

Protein Isolation and Nuclear Protein Fractionation

Muscle samples were collected via needle biopsy and homogenized on ice in Tissue Protein Extraction Reagent (Thermo-Fisher Scientific) in the presence of Halt protease/phosphatase inhibitors (Thermo-Fisher Scientific). The homogenate was centrifuged at 12,000xg for 5min at 4°C and then the supernatant was removed. Total protein per sample was quantified using a standard bicinchoninic acid (BCA) kit with bovine serum albumin (BSA) as the standard (Thermo-Fisher Scientific), and stored at -80°C for later analysis.

Nuclear fractions were isolated using previously described methods (5). Briefly, frozen muscle samples were homogenized in a low-salt buffer solution (10uM HEPES, pH 7.6; 10mM KCl; 1.5mM MgCl₂; 0.1mM EDTA; 0.1mM DTT; 0.5M PMSF; 50uL HALT protease/phosphatase inhibitor cocktail (Thermo-Fisher Scientific); and 0.5mg/ml benzamidine) and then incubated on ice for 5min. The homogenate was then cooled in an ethanol/dry ice bath for 5min, heated in a 37°C water bath for 1min, and vortexed briefly. This cooling and heating process was repeated and the samples were then centrifuged at 670 x g in a microfuge for 3min at 4°C. The supernatant, which contained the cytoplasmic proteins, was removed and stored at -80°C for later analysis. The remaining nuclear pellet was resuspended in an ice-cold high-salt buffer solution (20mM HEPES, pH 7.6; 420mM NaCl; 1mM EDTA; 25% glycerol; 1mM DTT; and 5uL protease inhibitor cocktail) and incubated on ice for 30min. After centrifugation at 12,560 x g for 5min at 4°C, the supernatant was removed and stored as the cytoplasmic protein. All protein samples (total and

nuclear) were quantified using a standard BCA kit (Thermo-Fisher Scientific) prior to the NFκB activity assay and Western blotting.

NFκB Activity

Components of the NFκB canonical (p65) and non-canonical (Rel-B) pathways were measured for DNA binding activity using a DNA-binding ELISA kit (Active Motif, Carlsbad, CA). All procedures were performed at room temperature. Nuclear fractions (12ug, in duplicate) were incubated in a 96-well plate containing a NFκB consensus sequence (5'-GGGACTTCC-3') for 1h. After washing, the plates were incubated for 1h with the primary antibody (1:1000) against p65 or Rel-B and then washed. The plates were then incubated with an HRP-conjugate secondary antibody (1:1000) for 1h and again washed. A developing solution was added, producing a colorimetric reaction that was allowed to develop over 5min. The reaction was halted with the addition of a stop solution and absorbance was read at 450nm with a correction wavelength of 655nm on a multi-well plate reader (FLUOstar Optima; BMG Labtech, Offenburg, Germany).

Western Blotting

Western blotting was used to quantify differences in protein levels of phosphorylated and total ERK between smokers and non-smokers and their responses to exercise. 40ug protein samples were loaded into a 4-15% TGX polyacrylamide gel (Bio-Rad, Hercules CA), and electrophoresis performed at 300V for 18min to separate proteins. These proteins were transferred for 60min at 50V

onto Immun-Blot polyvinylidene difluoride membrane (Biorad). Membranes were washed with Tris-buffered saline and 0.2% tween (TBS-T) and blocked with 5% milk in TBS-T for 1h at room temperature. After blocking, membranes were washed with TBS-T and incubated with primary antibody in blocking solution overnight at 4°C with rocking motion. Following primary antibody incubation, the membrane was washed and incubated at room temperature for 1h with HRP-conjugated secondary antibodies (Biorad). After washing, Supersignal West Pico enhanced chemiluminescence agent (Thermo-Fisher Scientific) was applied and blots imaged using a ChemiDoc XRS+ (Bio-Rad, Hercules CA). The blot was then stripped for 8min with Restore Western Blot Stripping Buffer (Thermo-Fisher Scientific), washed with TBS-T, and the process repeated for additional antibodies from the blocking step. The initial blot was probed for phosphorylated ERK (pERK, rabbit polyclonal, 1:2000, Cell Signaling, Danvers, MA, USA) followed by total ERK (ERK, rabbit polyclonal, 1:1000, Cell Signaling), and finally GAPDH, which served as a loading control (mouse monoclonal, 1:1000, Abcam, Cambridge, MA, USA). The resulting images were analyzed using ImageJ software (National Institutes of Health (NIH), Bethesda, MD, USA) (234).

Immunohistochemistry

Skeletal muscle samples collected from the control and exercised legs were analyzed for relative protein expression of myosin heavy chain-1 (MHC-1) tenascin-C (TNC), NGF, tyrosine receptor kinase A (TrkA) and p75 neurotrophin receptor (p75^{NTR}); and localization/co-localization of NGF, TrkA, and p75^{NTR} using

immunohistochemistry. Samples were embedded in Tissue-Tex O.C.T. compound (Sakura Finetek, Torrance, CA, USA), flash-frozen in liquid nitrogen, and stored at -86°C. 12µm cross-sections were generated on a Microm HM 505E cryostat at -20°C (Richard Allan Scientific, Kalamazoo, MI, USA) and mounted on Superfrost slides (Fisher Scientific, Pittsburgh, PA, USA). After air-drying for 30min, the sections were rehydrated for 5min with 1X PBS and 0.1% tween (PBS-T) for 5min at room temperature. After 15min blocking in 7% normal goat serum (Thermo-Fisher Scientific) at room temperature, sections were incubated overnight at 4°C with two primary antibodies simultaneously (as noted below) in PBS-T. The sections were then washed three times for 5min in PBS-T and incubated for 30min at room temperature with the appropriate secondary antibodies plus DAPI (1:100, Sigma, St. Louis, MO, USA). The slides were washed three times in PBS-T and once in ddH₂O for 5min each before being mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA). Sections were then imaged with a Nikon model TMS inverted microscope (Nikon, Tokyo, Japan) and a SPOT Insight imaging color camera (Diagnostic Instruments, Sterling Heights, MI, USA). Images were analyzed using ImageJ analysis software. The following primary antibodies were used: MHC-1 (mouse monoclonal, 1:3 dilution, Developmental Studies Hybridoma Bank, Iowa City, IA, USA); TNC (rabbit polyclonal, 1:100 dilution, EMD Millipore, Billerica, MA, USA); NGF (rabbit polyclonal, 1:100 dilution, Abcam); TrkA (mouse monoclonal, 1:50, Abcam); p75^{NTR} (mouse monoclonal, 1:50 dilution, Abcam). The following secondary antibodies were all used at 1:100 dilution: Dylight 488 goat anti-mouse iGg2b (for MHC-1; Jackson Immuno-Research Laboratories, West Grove, PA), Dylight

549 goat anti-rabbit (for MHC-1; Jackson Immuno-Research Laboratories, West Grove, PA), FITC-conjugated goat anti-mouse (TrkA and p75^{NTR}; Biorad), and TRITC-conjugated goat anti-rabbit (NGF).

Statistics

Unless otherwise noted above, data were analyzed statistically using a repeated-measures analysis of variance (RM ANOVA) (92), with time as the repeated factor and comparing the main effects of group (non-smokers vs smokers), exercise (control and exercise), and the group by exercise interaction. When baselines were different between groups due to an effect of smoking on non-exercised muscle, the baseline was applied as a covariate to investigate specific effects of the exercise on group responses. Tukey's HSD (345) was used to perform *post-hoc* analyses. Pearson product-moment correlations (274) were used to test correlations between protein and mRNA levels. Students T-Tests (322) were used to compare between groups for MHC content. Data were analyzed using SAS statistical software, and are presented as means \pm standard error, with significance between groups or conditions set at $p < 0.05$.

Results

***In Silico* Expression Profile Study**

Summary data for the *in silico* analyses are presented in Table 5.3. Of the 21 genes initially targeted for study using the GEO database, two (IL1A and IL1R1) were eliminated from consideration because smoking and/or pain were not

associated with changes to their expression. Of the remaining 19 targets selected for study, elevated expressions of four transcripts (CCL2, IL18, NGF, and TGFB1) were found in studies testing the effects of: 1) smoke exposure; and 2) pain. NGFR (p75^{NTR}) and IL1 β were elevated in some datasets but suppressed in at least one study after smoke exposure, indicating differential effects based on tissue or cell type. Both of these genes have been reported to enhance hyperalgesia through greater protein expression of IL1 β (28) or binding by NGF to either p75^{NTR} or TrkA (reviewed in Mizumura and Murase (224)). NTRK1 (TrkA), the high-affinity NGF receptor, was not affected by pain or smoking in any studies using the search criteria, although it is well established that binding with NGF produces hyperalgesia.

Twelve genes were differentially expressed after smoke exposure. Although associated with pain in other published works (30, 74, 75, 184, 262, 318, 330, 331, 344, 350, 367, 378, 384), expression of these genes was not associated with pain in the existing transcription profiles: cytokines CXCL1, CXCL2, CXCL10, IL6, IL6R, and IL18; TRAF6; ERK-related proteins MAP3K14 and MAP3K8; and NF κ B pathway components IKBKB, NFKBIA, and CHUK. Changes in the expression of cytokines CXCL1 and CXCL2 were equivocal (Table 5.3) while studies testing CXCL10, IL6, and IL6R showed elevated responses, only. Published reports indicate that IL-6 stimulates hyperalgesia (74, 75). TRAF6, which acts upstream of ERK and NF κ B, was elevated with smoke exposure in two published studies and may serve to maintain hyperalgesia (184). In the *in silico* analyses, smokers had elevated expression of ERK pathway components MAP3K14 (one study) and MAP3K8 (two studies)—activity of each protein result in downstream activation of ERK and NF κ B (MAP3K14 only).

IKBKB, an inhibitor of the canonical NFκB pathway, was suppressed in one study; another inhibitor, NFKBIA, was also suppressed in one study but elevated by smoking in five studies. Expression of CHUK, which activates the non-canonical NFκB pathway, was enhanced in two studies. Together, these data suggest an effect of smoking on NFκB activity, but the direction of this putative activity is unknown in muscle. Four of these genes (MAP3K14, MAP3K8, IKBKB, and NFKBIA) are not directly associated with hyperalgesia, although their downstream targets, ERK and NFκB, are associated with hyperalgesia (85, 111, 173, 196, 335, 355, 369, 373).

Together, the GEO profile analyses indicate that the interrelated NGF, ERK, and NFκB pathway activities may be altered in smokers. Given that these pathways are also involved in the post-exercise muscle response, hyperalgesia, and that smokers had greater DOMS in my previous study, I hypothesized that their activity would be affected by eccentric exercise. Specifically, I hypothesized that at 48h post-ECC: 1) activity of the canonical NFκB pathway would be suppressed in non-smokers but not in smokers, while the non-canonical (alternative) pathway would increase in non-smokers but not in smokers; 2) nuclear activity of the ERK pathway would increase for non-smokers while activity would be concentrated in the cytoplasm for smokers; and 3) NGF levels and binding with receptors would be more pronounced in smokers. Based on the GEO profile analyses, I validated key targets (including NFκB, ERK, and NGF) by subjecting non-smokers and smokers to ECC and tested muscle biopsy samples for mRNA and protein expression, protein activity, and protein localization.

Validation Study

Subjects

All subjects conformed to the study requirements and successfully completed the exercise protocol. One subject was removed from analyses after experiencing an intramuscular hematoma. Characteristics for the remaining subjects are presented in Table 5.4.

Creatine Kinase Activity

The two groups had similar plasma activity of creatine kinase (CK) prior to exercise, with pre-exercise levels of 150.4 ± 36.8 U/L for non-smokers and 122.3 ± 24.0 for smokers. The groups responded to the exercise similarly: increased activity post-exercise ($p < 0.05$) for both non-smokers (477.3 ± 85.4) and smokers (581.3 ± 140.8), with no significant group differences ($p = 0.4$).

Eccentric Exercise and Isometric Maximal Voluntary Contractions (MVC)

During the exercise session, non-smokers and smokers did similar amounts of work, with average work performed per repetition of 288.8 ± 5.7 for non-smokers and 282.9 ± 9.2 for smokers. There was an effect of exercise set, with a reduction of work produced as the sets progressed ($p < 0.05$). There was no interaction between set and smoking group ($p = 0.45$) or main effect of smoking status ($p = 0.2$). Peak eccentric torque also decreased over the course of the session with a significant interaction ($p < 0.05$) and main effect of set ($p < 0.05$), but no main effect of smoking status ($p = 0.16$). Smokers decreased peak eccentric torque more rapidly, while non-

smokers declined more gradually. However, when expressed as a percent of baseline (Figure 5.2), the significant effect of set remained ($p<0.05$), while the interaction ($p=0.10$) and group effect ($p=0.98$) did not reach significance.

Before exercise, there were no significant differences for baseline extension isometric strength (MVC, non-smokers: $264.6\pm14.3\text{N}\cdot\text{m}$; smokers: $234.9\pm17.2\text{N}\cdot\text{m}$, $p=0.2$). The exercise*smoking interaction effect was non-significant when data were expressed as raw numbers ($p=0.37$) or percent of pre-exercise levels ($p=0.4$), and there was no effect of smoking status (raw: $p=0.16$; percent baseline: $p=0.64$). There was a significant effect of exercise on both raw and normalized MVC ($p<0.05$). At 5min post-exercise, both groups experienced approximately 25-30% strength loss (Figure 5.3). At 4d after exercise, recovery was significantly suppressed for both groups ($p<0.05$; non-smokers: 1d $77.8\pm5.5\text{N}\cdot\text{m}$ vs 4d 78.6 ± 7.2 ; smokers: 1d $76.4\pm6.4\text{N}\cdot\text{m}$ vs 4d 68.7 ± 7.6). This may have been a side effect of the muscle biopsy, which was obtained at 48h post-exercise. At 9d, MVC were no longer significantly different from pre-exercise levels for both raw values ($p=0.59$) and percent of pre-exercise levels ($p=0.58$).

As with extension, there were no significant differences for flexion strength before exercise (MVC, non-smokers: 121.3 ± 8.3 ; smokers: 106.1 ± 4.0). The exercise*smoking interaction term was significant for both raw and data normalized to pre-exercise levels ($p<0.05$), and there was a significant main effect of smoking status for raw data ($p<0.05$) but not for exercise ($p=0.06$); when normalized to pre-exercise levels, the effect of smoking did not persist ($p=0.40$) while the effect of exercise was significant ($p<0.05$). Both the exercise*smoking interaction term and

exercise effect were driven by differences in exercise response at 4d post-exercise (2d post-biopsy), when non-smokers remained at pre-exercise levels while smokers decreased in flexion to approximately 81% of baseline. As with extension measures, this effect had resolved by 9d. These data are presented in Table 5.5.

PCR Arrays

mRNA quantification via PCR array are presented as fold change (Table 5.6, Supplemental), with significantly dysregulated genes shown in Figure 5.4. Expression for five genes was significantly different with exercise overall, with upregulation of AKT1, CDKN1A, and MAP2K1, and downregulation of PDK2 and VEGFA. There was no effect of group (smoking) for any gene of interest. There was a significant exercise*group interaction for three genes, such that smokers experienced a reduced fold change in comparison to non-smokers: ACTA1 (27% lower), FIGF (encodes for the VEGF-D protein, 50% lower), and PDPK1 (26% lower). Smokers had lower expression of these genes after ECC, while non-smokers displayed either baseline levels of expression or an increase. Similar expression patterns occurred after ECC (smokers down-regulated, non-smokers up-regulated or no change) for components of the ERK1/2 (MAP2K1, MAPK1, MAPK3) and NFκB (CHUK, NFKBIA, REL) pathways, although these did not reach the more stringent significance levels required when testing multiple genes.

qRT-PCR

The steady state levels of three transcripts identified previously (Dissertation Study 2, Chapter 4) were also analyzed by qRT-PCR: TRAF6, NFKBIA (an inhibitor of NFκB), and NGF (Figure 5.5). In the control leg, the expression of these genes did not differ between smokers and non-smokers, suggesting that there were no baseline differences in expression. TRAF6 is involved in the activation of numerous pathways, including ERK1/2 and NFκB. TRAF6 expression in response to exercise was not significantly different between groups ($p=0.16$) and did not differ in response to exercise ($p=0.35$) or between groups (0.52). Because TRAF6 activation stimulates ERK and NFκB activity and TRAF6 was upregulated early (3h) post-ECC, no difference in expression at 48h versus control may indicate a decrease in activation of ERK1/2, NFκB, and other related pathways by virtue of decreased TRAF6 expression. NFKBIA expression in response to exercise was similar between groups ($p=0.24$). Furthermore, NFKBIA expression was not significantly changed with exercise ($p=0.69$) or as a result of smoking status ($p=0.29$). The exercise response of NGF was also similar between smokers and non-smokers ($p=0.88$), and there was no overall effect of smoking ($p=0.93$). While both groups appeared to have higher NGF expression after exercise, this did not reach significance ($p=0.09$).

NFκB activity

Nuclear activity of the canonical (p65) and non-canonical (REL-B) NFκB pathways revealed differences between smokers and non-smokers in response to exercise (Figure 5.6). There were no differences in baseline (control leg) REL-B

activity between groups (3.8% lower in smokers). There was a significant interaction between exercise and smoking ($p < 0.05$): non-smokers experienced enhanced REL-B activity in the exercised leg (148% greater) while smokers failed to display a difference between exercised and control legs (102%). There was no overall effect of exercise on REL-B activity, although smokers had significantly lower REL-B activity (~21% lower, $p < 0.05$).

In the control leg, non-smokers had a higher level of p65 activity as compared to smokers (60% greater in non-smokers, $p < 0.05$). In response to the exercise, smokers and non-smokers differed significantly in p65 activity ($p < 0.05$). When compared to control, non-smokers had considerably lower p65 activity in the exercised legs (14% of control), whereas smokers did not differ significantly between legs (exercise leg: 6% lower than control). The overall effect of exercise on p65 activity did not reach significance ($p = 0.07$), and there was no overall effect of smoking status.

ERK Activity

ERK is activated through phosphorylation (pERK), which in turn activates distinct signaling pathways in the cytosol and nucleus. The amount of total ERK, and therefore the total capacity for activation, generally remains unchanged in response to a variety of stimuli and is often used as a secondary control for ERK activity (ERK activity ratio). However, total ERK may differ between smokers and non-smokers. Both pERK and total ERK protein levels were normalized to the loading control GAPDH. Because pERK and total ERK were probed using different antibodies, their

values could not be compared directly. However, normalization relative to GAPDH is possible, and these normalized data are discussed below and presented in Figure 5.7.

Exercise did not affect the relative levels of pERK or total ERK—overall or as an interaction with smoking. In nuclear fractions, there was greater total (45%, $p<0.05$) and pERK (290%, $p<0.05$) compared to cytosol, which did not differ with exercise. Smokers had lower total ERK than non-smokers, specifically in the nuclear compartment (38% of non-smokers, $p<0.05$). Therefore, the ERK activity ratio could not be used; rather, total ERK was included as a covariate in the pERK statistical model, yielding pERK_{total} (the amount of pERK relative to total ERK levels).

Without total ERK as covariate, the sole significant finding was lower cytosolic pERK in smokers when compared to non-smokers (59% of non-smokers, $p<0.05$). When expressed as pERK_{total}, this no longer met significance criteria ($p=0.09$). Yet differences in pERK_{total} indicated that smokers had greater ERK activity *relative to the total amount of ERK available* in the nuclear compartment than non-smokers ($p<0.05$).

Together, these data indicate that smokers had a lower capacity for ERK activity, specifically in the nucleus, yet used a greater percentage of available ERK for signaling within the nucleus. However, there was no difference in overall pERK between groups in the nucleus ($p=0.37$). Smokers did have less pERK in the cytosol ($p<0.05$), indicating that there may be a general suppression of cytosol-specific ERK signaling.

Immunohistochemistry

Sample availability was limited for immunohistochemical analyses so samples from only three non-smokers and four smokers were used to test the following parameters: 1) fiber type distribution via myosin heavy chain-I; 2) muscle damage via tenascin-c expression; 3) protein levels of NGF and its receptors, TrkA and p75^{NTR}; and 4) co-localization of NGF and each of its receptors (TrkA and p75^{NTR}).

Fiber Type Distribution: Myosin Heavy Chain-I

Anti-MHC-I immunofluorescence analysis of skeletal muscle sections revealed that there were differences in fiber type composition between groups ($p < 0.05$): non-smokers had significantly greater fibers positively stained for MHC-I (Type I oxidative) than smokers ($p < 0.05$, Figure 5.8). Both groups had similar numbers of fibers (non-smokers: 183 ± 17 fibers; smokers 202 ± 25 fibers), but while non-smokers had an approximately equal fiber type distribution, smokers had 13% lower MHC-I-positive fibers.

Tenascin-C

Tenascin-c (TNC) was used as an indicator of muscle damage and remodeling. It appeared that smokers had greater TNC levels post-exercise (non-smokers control vs exercise: 5276 ± 1701 pixels vs 15917 ± 5630 ; smokers: 2700 ± 1195 pixels vs 23150 ± 12732 , $p = 0.88$, Figure 5.9). However, this was a small sample size with high variability for TNC expression, and the exercise response did

not differ significantly between smokers and non-smokers, nor was there a significant effect of group. TNC levels were, however, significantly higher after exercise in both groups ($p < 0.05$), indicating that the exercise was sufficient to elicit damage.

NGF, TrkA, and p75^{NTR}

NGF localized primarily to the muscle membrane, although staining of some nuclei was evident (Figure 5.10, center). There were no significant effects of smoking or exercise, and no significant interaction between the two (Figure 5.12a). When data from control and exercised muscle were combined, differences between smokers and non-smokers remained insignificant ($p = 0.10$). NGF elicits different responses based on which of two receptors it binds, and therefore differences in the number of receptors and co-localization may contribute to DOMS and other downstream signaling. Therefore, I examined the expression of the low-affinity receptor p75^{NTR} and the high affinity receptor TrkA and their presumptive binding to NGF using co-localization.

Low-level signal of TrkA appeared to be localized to the membrane (Figure 5.10, right). There was a significant interaction ($p < 0.05$) between smoking and exercise, indicating that smokers had greater levels of TrkA after ECC, while non-smokers had slightly lower levels after ECC (Figure 5.12a). There was no overall effect of exercise or group. TrkA and NGF were co-localized (Figure 5.10, left; Figure 5.12b) in 25% of the total signal volume of the sections for both groups. Roughly 55% of total TrkA signal was co-localized with NGF, and 57% of total NGF signal was

co-localized with TrkA. There were no significant differences in co-localization of these proteins with exercise, smoking, or their interaction, and the co-localization correlation was weak for exercise (non-smokers: $r=0.31$; smokers: $r=0.32$) and moderate for control (non-smokers: $r=0.44$; smokers: $r=0.50$).

The tissue distribution of p75^{NTR} was similar to that of NGF (Figure 5.11, center). There was no interaction between exercise and smoking and no effect of exercise for p75^{NTR} expression. However, smokers had significantly greater p75^{NTR} expression overall ($p<0.05$, Figure 5.12a). There was a significant interaction ($p<0.05$) between smoking and exercise for the percent volume of co-localization between p75^{NTR} and NGF (Figure 5.11a, Figure 5.12c). Co-localization of p75^{NTR} and NGF in non-smokers was 40% of the total signal volume in the control leg and decreased to 31% in the exercised leg. Compared to non-smokers, co-localization of p75^{NTR} and NGF was greater in the control leg (59% of total signal volume) and increased to 72% in the exercise leg. Overall, co-localization did not differ significantly between smokers and non-smokers ($p=0.07$). There was an overall effect of smoking on the percent of total signal co-localized for p75^{NTR} ($p<0.05$) and NGF ($p<0.05$). In control legs, 74% of total p75^{NTR} and NGF signals were co-localized in non-smokers, with 86% co-localization in smokers. In the exercised legs of non-smokers, 56% of total p75^{NTR} signal co-localized with 70% of NGF signal; for smokers, this co-localization was 93% for p75^{NTR} and 90% for NGF. The co-localization correlation was strong for control and exercise in both groups (control, smokers and non-smokers: $r=0.70$; exercise: non-smokers $r=0.73$, smokers $r=0.66$).

NGF is a secreted factor that can translocate into the cytoplasm via endosomal transportation and may play a role in the nucleus as a transcription factor in conjunction with other signaling molecules. In this study, I found significant co-localization of NGF with nuclei that decreased after exercise ($p < 0.05$, data not shown). There was also a significant interaction between smoking status and exercise: after ECC, non-smokers experienced a significant decrease in NGF-nuclear co-localization ($p < 0.05$), while the decrease for smokers did not reach significance (control: non-smokers, 6.8% total signal volume; smokers, 5.1%; exercise: non-smokers, 4.0%; smokers, 3.5%). These are preliminary findings, however, that require additional study to determine the specific cell types in which this co-localization occurs.

Discussion

This study tested mechanisms hypothesized to explain greater muscle fatigue and DOMS in smokers after ECC (Dissertation Study 1, Chapter 3), specifically if: 1) REL-B activity and fiber type distribution differed between muscle of smokers and non-smokers (fatigue); and 2) NF κ B and ERK activity, along with NGF protein levels, were altered in smokers (DOMS). I hypothesized that: 1) smokers would have a greater proportion of Type II fibers and suppressed REL-B activity at 48h post-ECC compared to non-smokers; 2) after ECC the activity of the canonical NF κ B pathway would be suppressed in non-smokers but not in smokers; 3) nuclear activity of the ERK pathway would increase for non-smokers while activity would be concentrated in the cytoplasm for smokers; and 4) NGF levels and receptor co-localization would

be more pronounced in smokers versus non-smokers. To my knowledge, this was the first study to investigate the effects of chronic cigarette smoking on molecular responses of skeletal muscle to ECC in humans.

I hypothesized that smokers would have a greater ratio of Type II:Type I muscle fibers and suppressed REL-B activity when compared to smokers. In the current study, histochemical analysis revealed a greater prevalence of Type II muscle fibers in smokers (~60% total fibers), while non-smokers had ~1:1 ratio of Type I and Type II fibers. While still equivocal, other studies have reported similar fiber type shifts with cigarette smoke exposure (72, 162, 169, 290, 328). Other potential mechanisms underlying muscle fatigue in smokers have been reviewed in greater detail previously (Dissertation Study 1, Chapter 3). I provide evidence that supports the sparse published research linking smoking to muscle fatigue and fiber type shift and, further, provide a potential mechanism: REL-B pathway activation post-ECC (which, among its functions, promotes oxidative metabolism (12, 13)), was suppressed in smokers. Shifts in fiber type distribution (greater Type II than controls) in response to smoke exposure (162, 328) and greater muscle fatigue (328) have been associated with suppressed PGC-1 expression. The PGC-1 protein family promotes the development of Type I muscle fibers (12, 328) and can be activated by REL-B, and, therefore, the suppressed REL-B response in smokers may attenuate PGC-1 signaling and lead to a predominance of Type II fibers.

Given that smokers had fewer Type I oxidative fibers and, in Dissertation Study 1 (Chapter 3), smokers experienced less fatigue resistance during ECC than non-smokers, my data suggest a mechanism through which smoking promotes a

fiber type shift in favor of more fatigable Type II fibers. This fiber type distribution could predispose smokers to greater risk for injury: smokers may rely on other, weaker muscles and/or muscles may fail to perform tasks safely. Type II fibers are more susceptible to injury, possibly through lower fatigue resistance, greater potential loads imposed upon these fibers, or other as of yet unknown mechanisms. These data therefore may explain the greater risk for injury in smokers. Smoking-suppressed REL-B activity in non-muscle cells is also associated with increased pro-inflammatory signaling including COX2 activity, while overexpression of REL-B rescues cigarette smoke-induced pro-inflammatory responses and reduces COX2 and prostaglandin E₂ expression(218). These data indicate that suppressed REL-B activity in smokers may also contribute to DOMS in smokers.

Based on previously published reports and findings from Dissertation Study 2 (Chapter 4), I hypothesized that the canonical NFκB pathway would be suppressed in non-smokers, but not in smokers. In Dissertation Study 2, p65 DNA binding activity (pro-inflammatory) was suppressed at 48h post-ECC in non-smokers. In the current study, p65 activity did not change after ECC in smokers. This finding is novel, as NFκB activity had yet to be tested in the skeletal muscle of smokers after ECC. Skeletal muscle activity of the pro-inflammatory canonical NFκB pathway has been tested in only a small handful of studies, with equivocal results (119, 291). Recent research has shown increased NFκB activity *in vitro* during proliferation of myoblasts that decreases during differentiation (13), and enhanced activity early (3h) after ECC (137). Enhanced activity of the canonical pathways is well established in response to smoking, although it had not yet been evaluated in

skeletal muscle of smokers. p65 is considered a keystone of inflammation, the activity of which promotes pro-inflammatory signaling. The current data suggest a lack of pro-inflammatory resolution at 48h in smokers, which may negatively impact healing and adaptation. Failed inflammatory resolution could also prolong hyperalgesic signaling.

ERK stimulates gene expression (including anti-inflammatory genes) and can affect canonical NF κ B pathway activity in the cytoplasm. Therefore I hypothesized that nuclear ERK activity would increase for non-smokers and remain concentrated in the cytoplasm for smokers. Because pERK was not affected by ECC at 48h, these data do not explain suppressed p65 activity in non-smokers. pERK may have suppressed p65 activity before 48h, yet this change in activity could have resulted through other mechanisms. In the current study, pERK was suppressed in the cytosol of smokers (both legs). Cigarette smoke extract or components, including nicotine, invoke rapid and transient activation of ERK1/2 in non-muscle tissue (177, 243). The majority of studies report transient (~15min) ERK activation after smoke exposure, yet sustained changes to activity (333) may occur. In the current study smokers were required to have a cigarette 10min prior to the muscle biopsy procedure (>30-40min before sample collection), indicating that, after transient activation, smoking may induce chronic pERK suppression. Smokers also had lower total nuclear ERK compared to non-smokers. To my knowledge, only one published study has reported changes to total ERK protein levels (144). Lower total ERK may limit the capacity for ERK activity; therefore, to maintain normal nuclear ERK signaling (indicated by similar nuclear pERK), smokers must activate a greater

proportion of available ERK. Thus, smokers may have a lower ceiling for the extent of pERK activity and so when a more substantial ERK response is required, be unable to meet the demands.

I originally postulated that NFκB and ERK affect hyperalgesia and, therefore, might contribute to greater DOMS experienced in smokers. It is possible that differences in NFκB and ERK activity early after ECC affected hyperalgesia, yet their early activity was not tested. NFκB activity did remain elevated compared to non-smokers, and so may contribute to greater DOMS in smokers. However, the lack of change in ERK activity in smokers at 48h, coupled with its suppressed activity compared with non-smokers, suggests that ERK may not play a significant role in greater DOMS reported by smokers.

NGF, which promotes hyperalgesia, is elevated in smokers, and so I hypothesized that levels of NGF and co-localization with its receptors would be more pronounced in smokers than non-smokers. An important factor in the development of hyperalgesia and specifically DOMS (reviewed in (224)), NGF exerts disparate downstream signaling by bindings to TrkA (ERK1/2) and p75^{NTR} (canonical NFκB pathway) receptors, and both are implicated in hyperalgesia. Although evidence better supports TrkA in this role, p75^{NTR} also can stimulate hyperalgesia (155). NGF can also bind simultaneously with p75^{NTR} and TrkA and stimulate hyperalgesia (reviewed in (20)). Due to technical limitations, the co-localization of NGF with both TrkA and p75^{NTR} was not possible in the current study.

In the current study, NGF was unaffected by smoking or exercise. While a limited sample, increased TrkA expression with exercise in smokers may indicate a

greater potential for NGF binding. However, co-localization with NGF was unchanged, suggesting that signaling downstream of TrkA was not affected. However, neurotrophin 3 (NTF-3), another neurotrophin, can bind, less efficiently than NGF, to TrkA and the location of that binding can either agonize or antagonize NGF effects (reviewed in (20)). It is therefore possible that, after ECC, NTF-3 also binds TrkA in smokers and this binding enhances its hyperalgesia. Future study should therefore include co-localization of these neurotrophins to TrkA.

Smokers experienced greater overall p75^{NTR} expression and NGF co-localization with p75^{NTR} than TrkA in the current study. Further, co-localization decreased in non-smokers with exercise, and increased in smokers—indicating a mechanism through which smoking could result in greater DOMS. Greater expression of p75^{NTR} and co-localization with NGF suggest a “priming” effect, whereby nociceptors are already at a lower activation threshold. These nerves would respond more intensely to NGF and other signals, such as COX2.

Unfortunately, the limited samples available for analysis did not allow me to investigate specifically the expression, localization, and co-localization of NGF and its receptors to nerves within the muscle sections, and so specific effects of smoking to the nerve signaling of these proteins must be left for future study. Smoking likely enhances nerve sensitivity through multiple mechanisms: for example, COX2 signaling (6) and transient receptor potential vanilloid 1 (TRPV1)(102), other hyperalgesic mediators, may play a role in greater DOMS in smokers and therefore additional study is needed. However, data from Dissertation Study 2 (Chapter 4) and the current study do not support those pathways. Rather, these data, specifically the

increase in NGF and p75^{NTR} co-localization after ECC in smokers, provide a mechanism through which DOMS is enhanced in smokers, possibly through continued NFκB activation (rather than suppression, as seen in non-smokers) at 48h after ECC.

In summary, I found that smokers had a greater percentage of the less fatigue resistant Type II fibers, which may be a result of attenuated activation of the non-canonical (REL-B) NFκB pathway. Smokers also had suppressed ERK signaling and canonical (p65) NFκB activity, as well as a lack of NFκB response to ECC. These signaling differences may attenuate proliferation and muscle regeneration, prolong pro-inflammatory signaling, and, potentially, suppress inflammatory resolution and healing. p75^{NTR} levels were greater in smokers, as was its co-localization with NGF, which increased after ECC. Taken together, this Dissertation suggests several mechanisms through which smokers are predisposed to greater muscle fatigue, soreness, and susceptibility to musculoskeletal injury.

Tables and Figures

Table 5.1: *In Silico* Expression Profile Analysis.
See Supplemental page 3

Figure 5.1: Study Timeline.

Blood was drawn at 2 visits, maximal voluntary contractions (MVCs; strength measure) were tested at 4 visits with a familiarization session at the initial visit, and eccentric exercise and biopsies were performed at one visit, each.

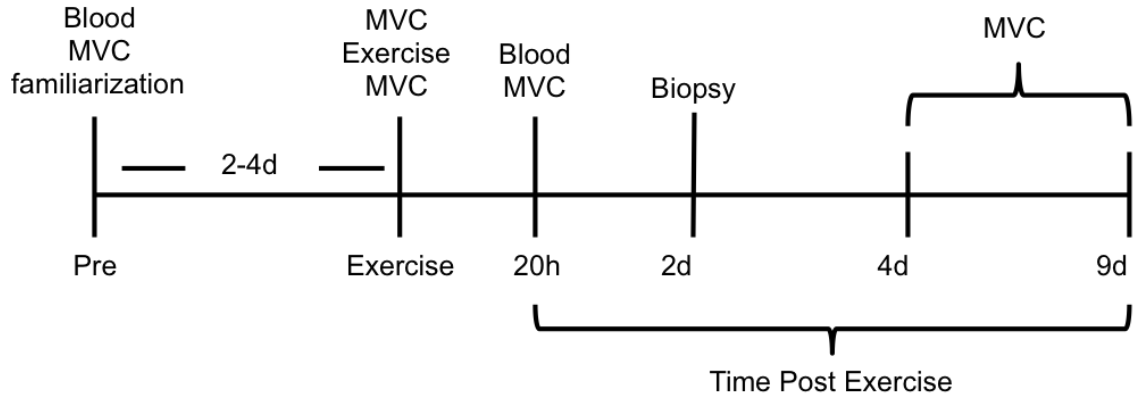


Table 5.2: qRT-PCR Primers.

NGF, nerve growth factor; NFKBIA, Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; TRAF6, TNF receptor-associated factor 6; B2M, Beta-2-microglobulin.

Target	Primer	Reference
NGF	Fwd: 5'- CAACAGGACTCACAGGAGCA -3'	(2)
	Rev: 5'- ACCTCTCCCAACACCATCAC -3'	
NFKBIA	Fwd: 5'-TGG CCT TCC TCA ACT TCC AGA ACA-3'	(137)
	Rev: 5'-CTC AGC AAT TTC TGG CTG GTT GGT -3'	
TRAF6	Fwd: 5'-TGATAGTGTGGGTGGAAGT-3'	(282)
	Rev: 5'-CTCCTTGGACAATCCTTCAG-3'	
B2M	Fwd: 5'-TGTCTGGGTTTCATCCATCCGACA-3'	(137)
	Rev: 5'-TCACACGGCAGGCATACTCATCTT-3'	
	Rev: 5'-TCACACGGCAGGCATACTCATCTT-3'	

Table 5.3: Subject Characteristics.

There were no significant differences between groups for height, weight, body mass index (BMI), or age. Data are presented as mean \pm SEM.

	N	Age (y)	Height (cm)	Weight (kg)	BMI
Non-smokers	9	22.0 \pm 0.8	180.7 \pm 3.3	82.2 \pm 2.8	25.2 \pm 0.7
Smokers	10	23.3 \pm 1.2	173.2 \pm 2.0	75.0 \pm 3.9	25.0 \pm 1.2

Figure 5.2: Peak Eccentric Torque During Exercise.

Non-smokers —●— ; smokers --□-- . Data are presented as mean \pm SEM. * p<0.05 effect of exercise (compared to set 1).

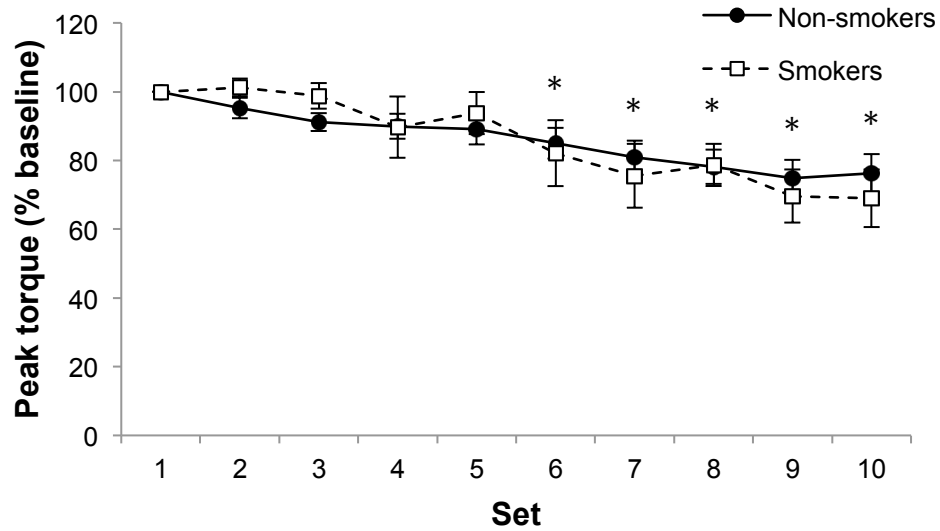


Figure 5.3: Isometric Extension Maximal Voluntary Contraction.

Non-smokers ■; smokers □. Data are presented as a percent of pre-exercise levels and are mean±SEM. * p<0.05 effect of exercise (compared to pre-exercise).

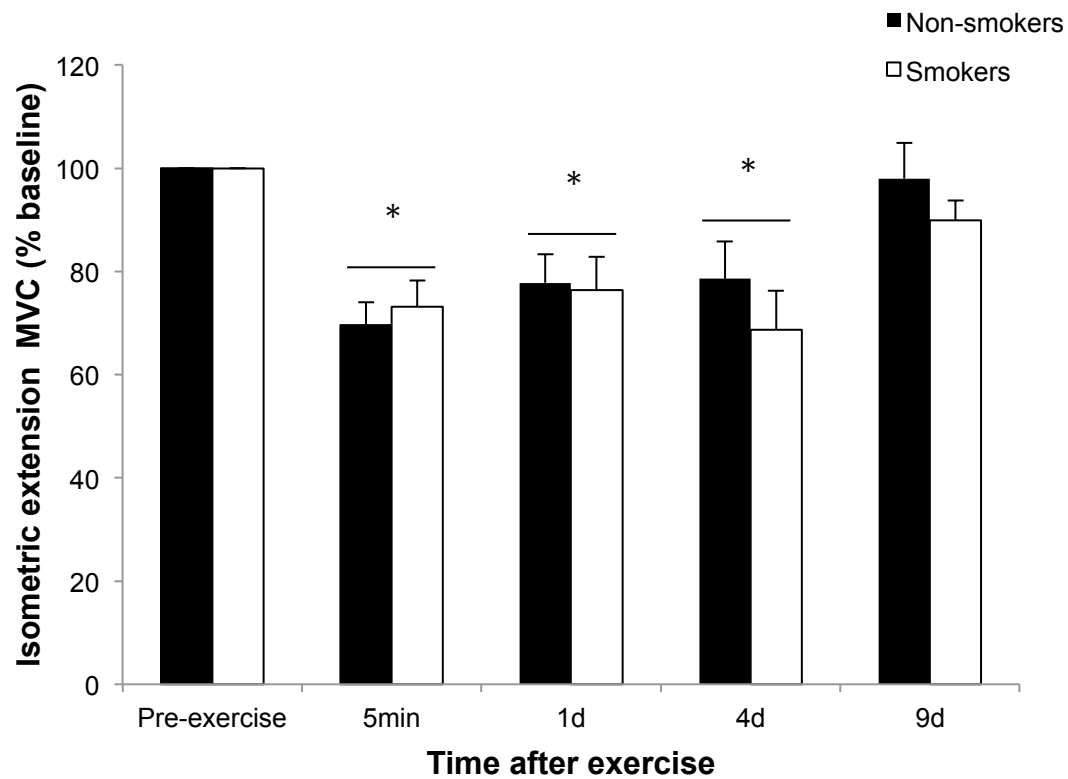


Table 5.4: Isometric Flexion Maximal Voluntary Contraction.

Data are presented as N•m, and are mean±SEM. * p<0.05 effect of exercise; † p<0.05 effect of group (smoke).

	Time Post-exercise				
	Pre-exercise	5min	1d	4d	9d
Non-smokers	121.3±8.3	112.8±8.0	112.0±4.7	120.8±56.9*	128.1±5.0
Smokers	106.1±4.0	107.3±47.0	107.1±5.9	86.1±8.1* †	106.2±8.2

Table 5.5: Gene Expression after Eccentric Exercise.

Arrays were comprised of 44 genes of interest, normalized to two housekeeping genes. Gene names and symbols from the National Institute of Health (NIH) National Center of Biotechnology (NCBI) database, June 2015. Functions from NCBI, UniProt, Genecards, and published literature. Data are presented as fold change (exercise vs control leg) and were analyzed as $\Delta\Delta C_t$ using repeated measures ANOVA, with significance based on false discovery rate (FDR): * $p < 0.007$, effect of exercise; § $p < 0.003$, interaction (group*exercise). No genes were significant ($p < 0.002$) for the effect of group (smoke).

See Supplemental page 4

Figure 5.4: PCR Array—Expression Affected by Exercise.

Non-smokers ■; smokers □. Data are presented as fold-change (mean±SEM) and were analyzed (as $\Delta\Delta C_t$) using repeated measures ANOVA, with significance based on false discovery rate (FDR): * $p < 0.007$, effect of exercise; § $p < 0.003$, interaction (group*exercise).

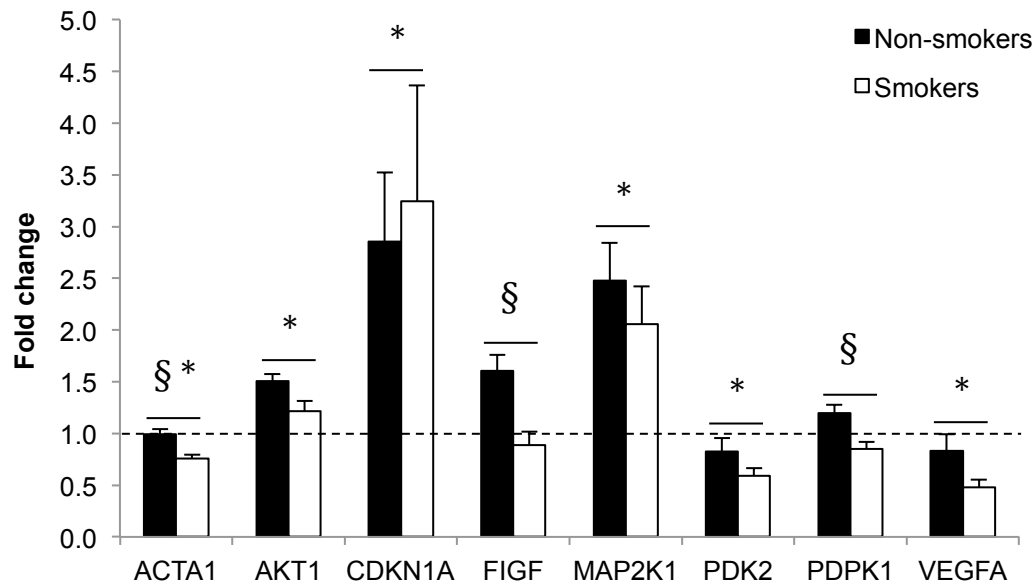


Figure 5.5: qRT-PCR Analyses—TRAF6, NFKBIA, and NGF.

Non-smokers ■; smokers □. Data are presented as fold change (mean±SEM). There were no significant effects of group or time. NGF, nerve growth factor; NFKBIA, nuclear factor kappa B inhibitor-alpha; TRAF6, TNF-associated factor 6.

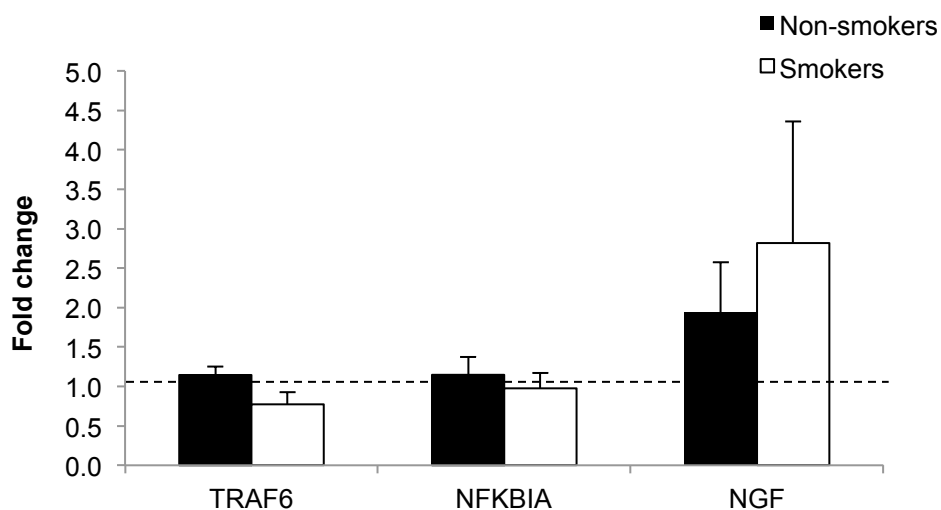


Figure 5.6: NFκB Activity Changes with Eccentric Exercise.

Activity of (a) the canonical p65 pathway; and (b) the non-canonical REL-B pathway at 48h after ECC. Control ■; exercise □. § $p < 0.05$, interaction (group*exercise); † $p < 0.05$, effect of group (smoke). NFκB, nuclear factor kappa beta; p65 (REL-A), v-rel avian reticuloendotheliosis viral oncogene homolog A; REL-B, v-rel avian reticuloendotheliosis viral oncogene homolog B.

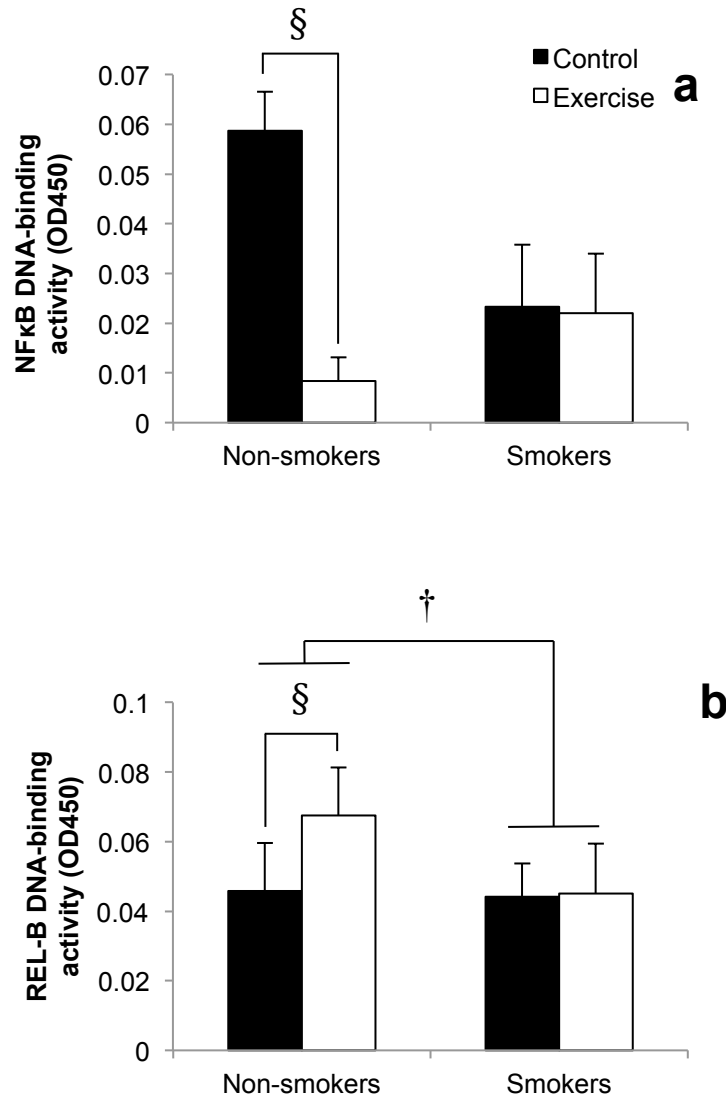
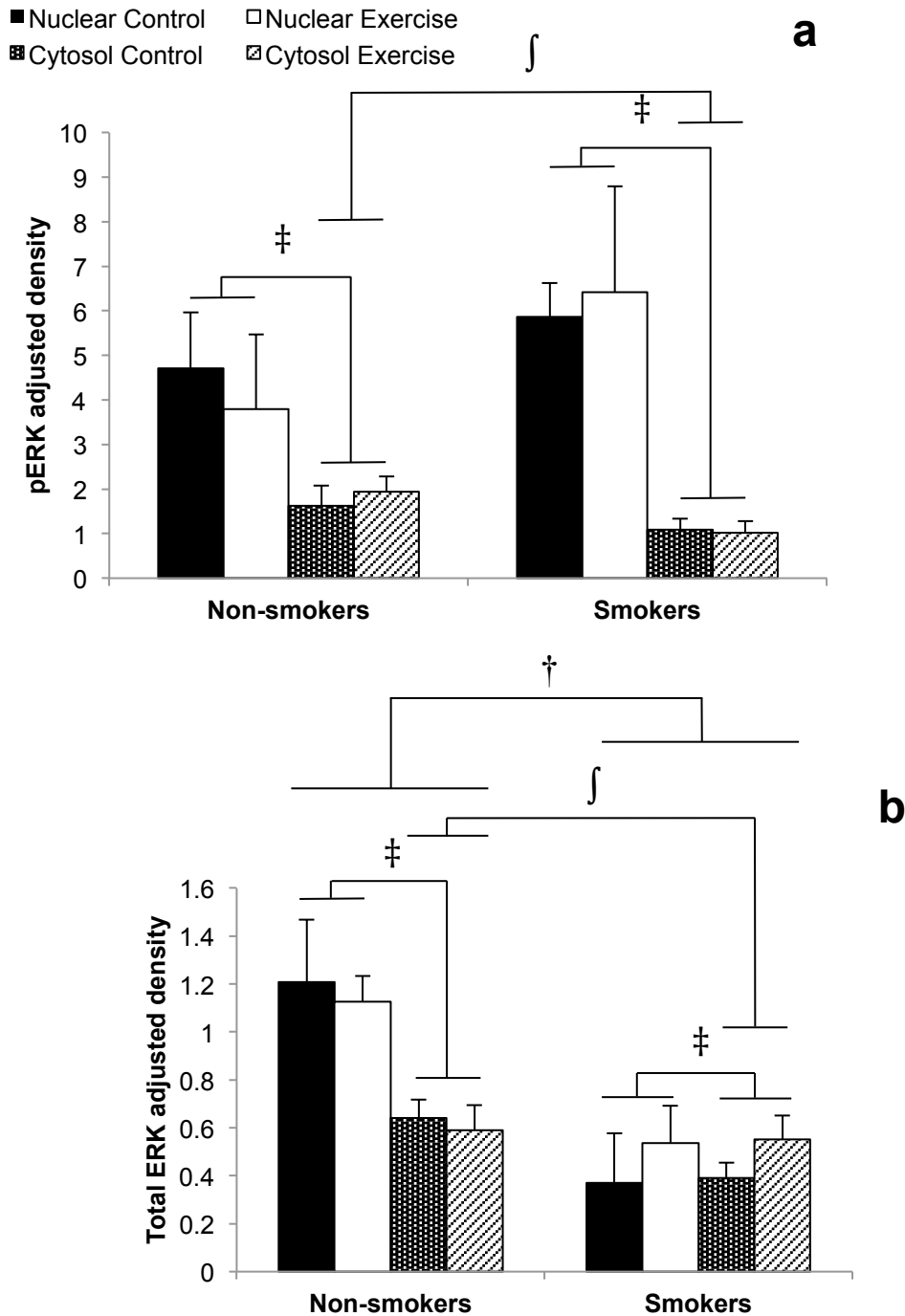


Figure 5.7: ERK Activity.

pERK levels (a) indicate the amount of activated ERK, and total ERK levels (b) indicate the capacity for ERK activity. Nuclear controls ■; nuclear exercise □; cytoplasm nucleus ▨; cytoplasm exercise ▩. Representative blots (next page) of nuclear (c) and cytosol (d) fractions are shown. Non-smokers are at left and smokers at right, alternating control and exercise across lanes. ∫ p<0.05, interaction (group*fraction); † p<0.05, effect of group (smoke); ‡ p<0.05, effect of fraction.



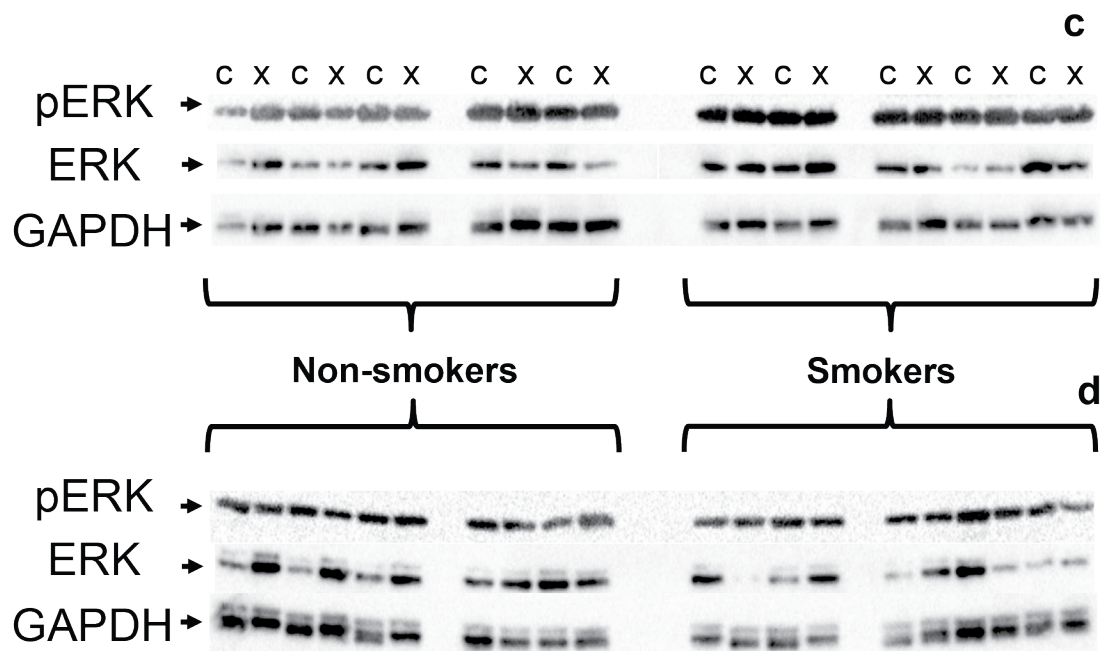


Figure 5.8: Myosin Heavy Chain Isoform Distribution.

The percent of total fiber counts positive (□) or negative (■) for MHC-I are shown (a), with average fiber counts per section displayed. Representative images of cross sections at 12uM for non-smokers (b) and smokers (c) are shown, with MHC-I positive fibers in green and nuclei stained blue (DAPI). † p<0.05, effect of group (smoke). MHC, myosin heavy chain; DAPI, DAPI, 4',6-diamidino-2-phenylindole.

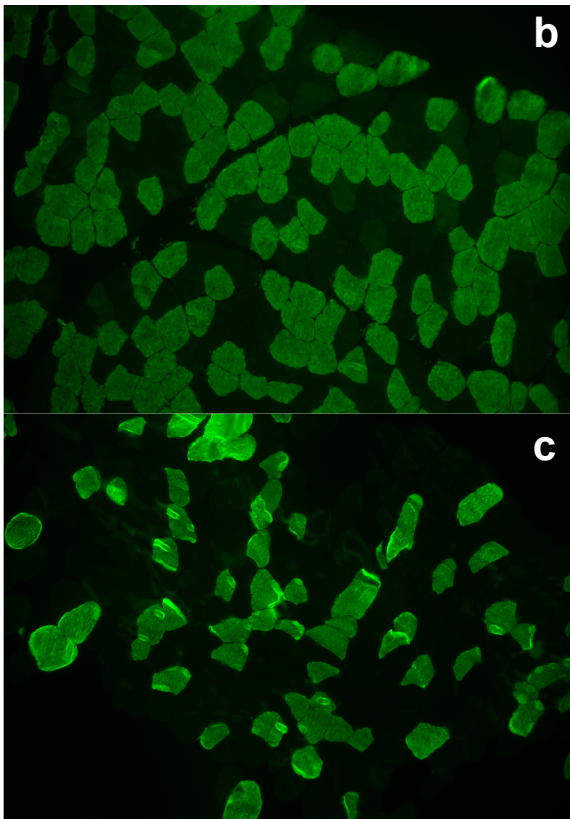
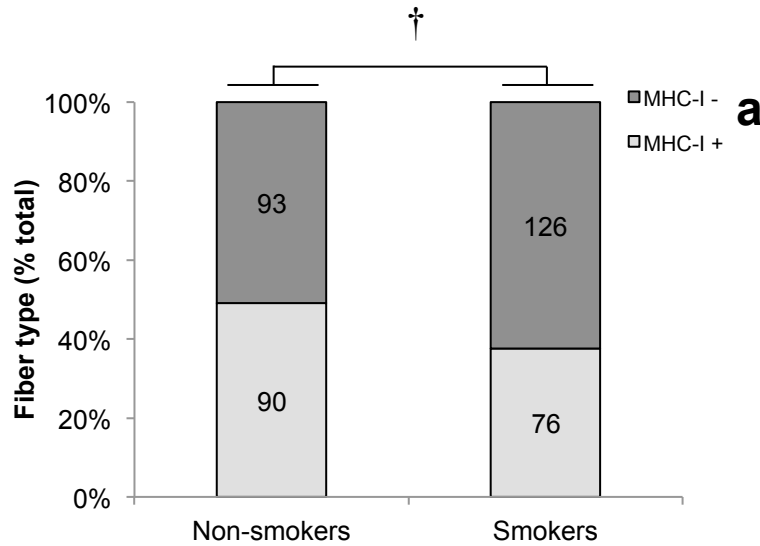


Figure 5.9: Tenascin-c Immunofluorescence.

The area of TNC immunofluorescence, indicating protein expression, is shown in control (■) and exercised (□) legs at 48h after eccentric exercise. Representative images of cross sections at 12uM for TNC (red) are shown for non-smokers (b: control; c: exercise) and smokers (d: control; e: exercise). * $p < 0.05$ effect of exercise. TNC, tenascin-c.

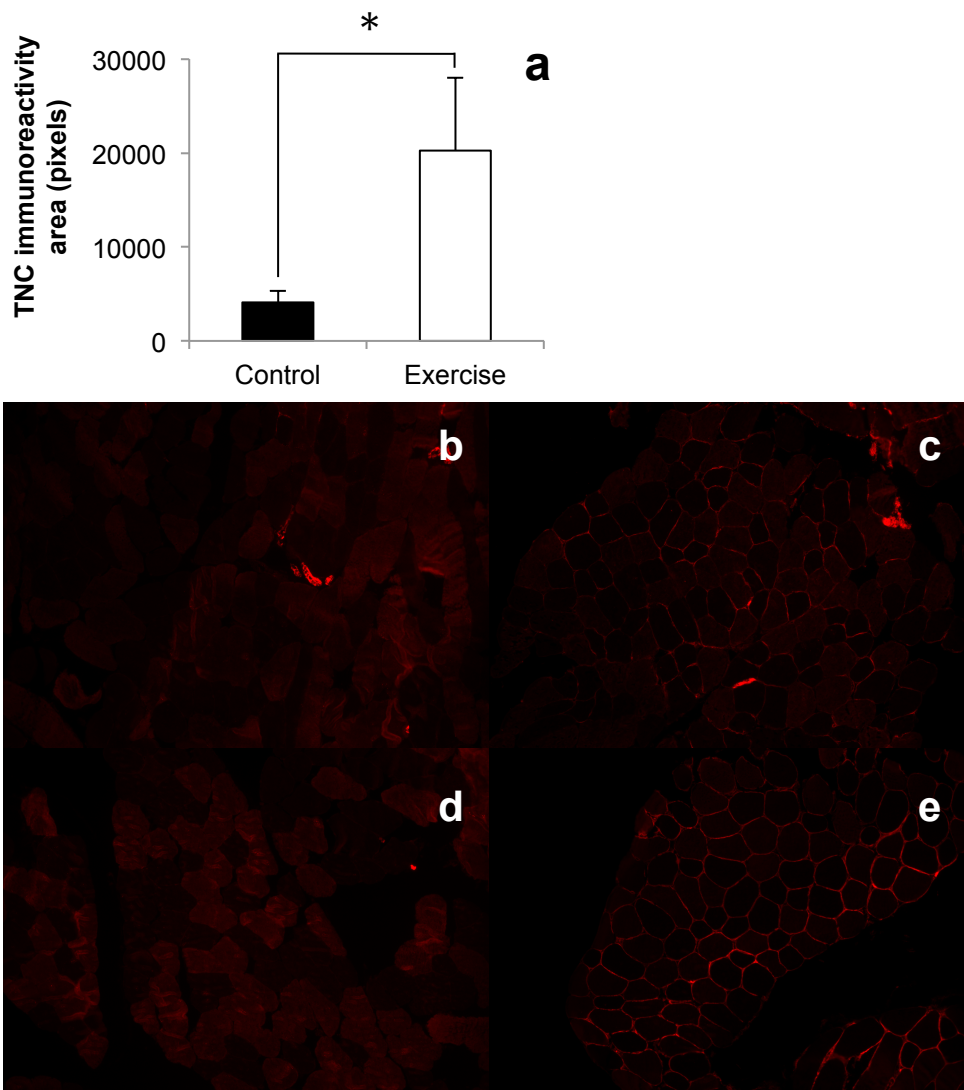


Figure 5.10: NGF and TrkA Co-Localization.

Representative images of muscle cross-sections at 12uM: non-smoker (control: a-c; exercise: d-f) and smoker (control: g-i, exercise: j-l). Co-localization are shown far left (a, d, g, j), NGF in center (green, b, e, h, k), and TrkA at right (c, f, i, l). NGF, nerve growth factor; TrkA, tyrosine receptor kinase A; DAPI, 4',6-diamidino-2-phenylindole.

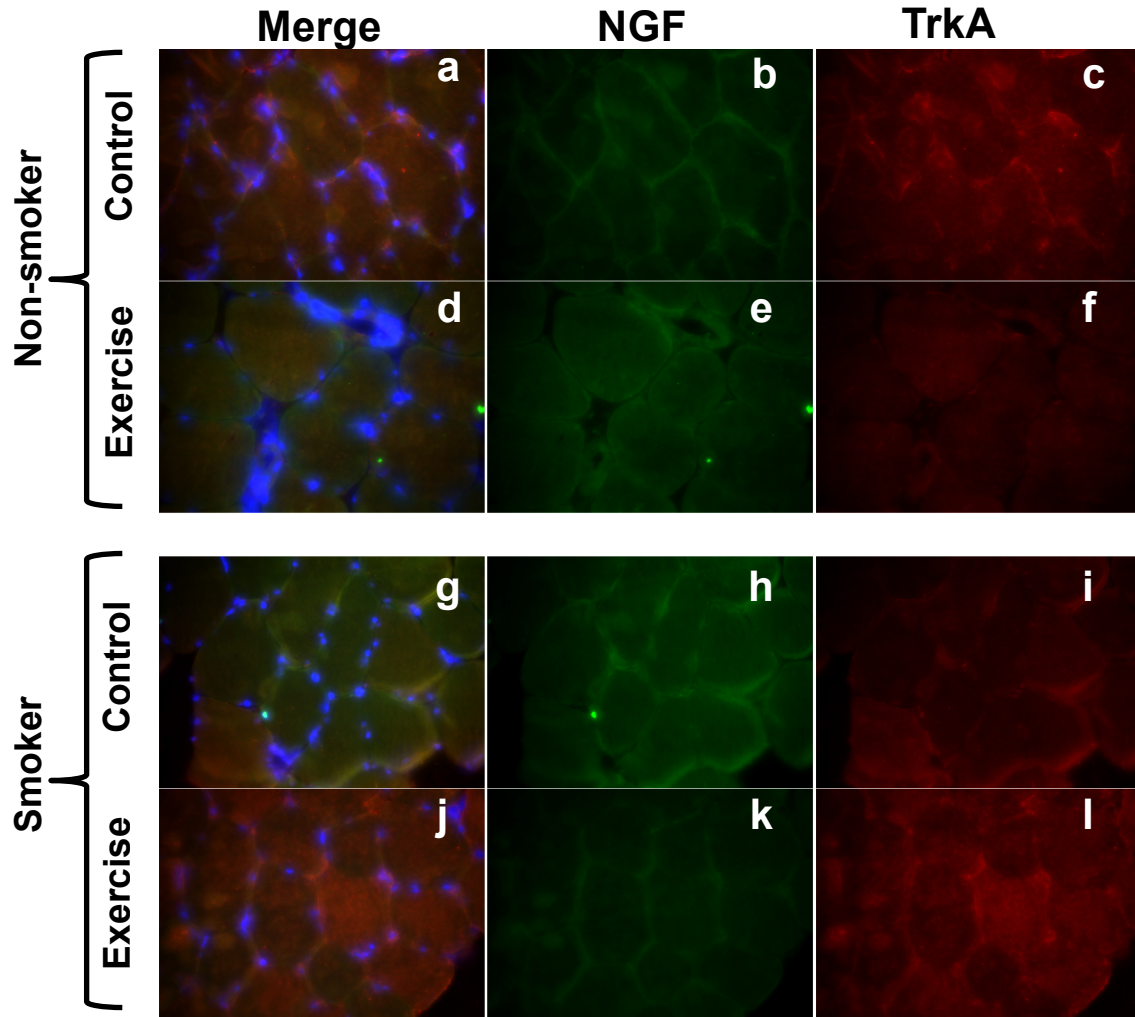


Figure 5.11: NGF and p75^{NTR} Co-Localization.

Representative images of cross sections at 12uM: non-smoker (control: a-c; exercise: d-f) and smoker (control: g-i, exercise: j-l). Co-localization are shown far left (a, d, g, j), NGF in center (green: b, e, h, k), and p75^{NTR} at right (red: c, f, i, l). NGF, nerve growth factor; p75^{NTR}, p75 neurotrophin receptor; DAPI, 4',6-diamidino-2-phenylindole.

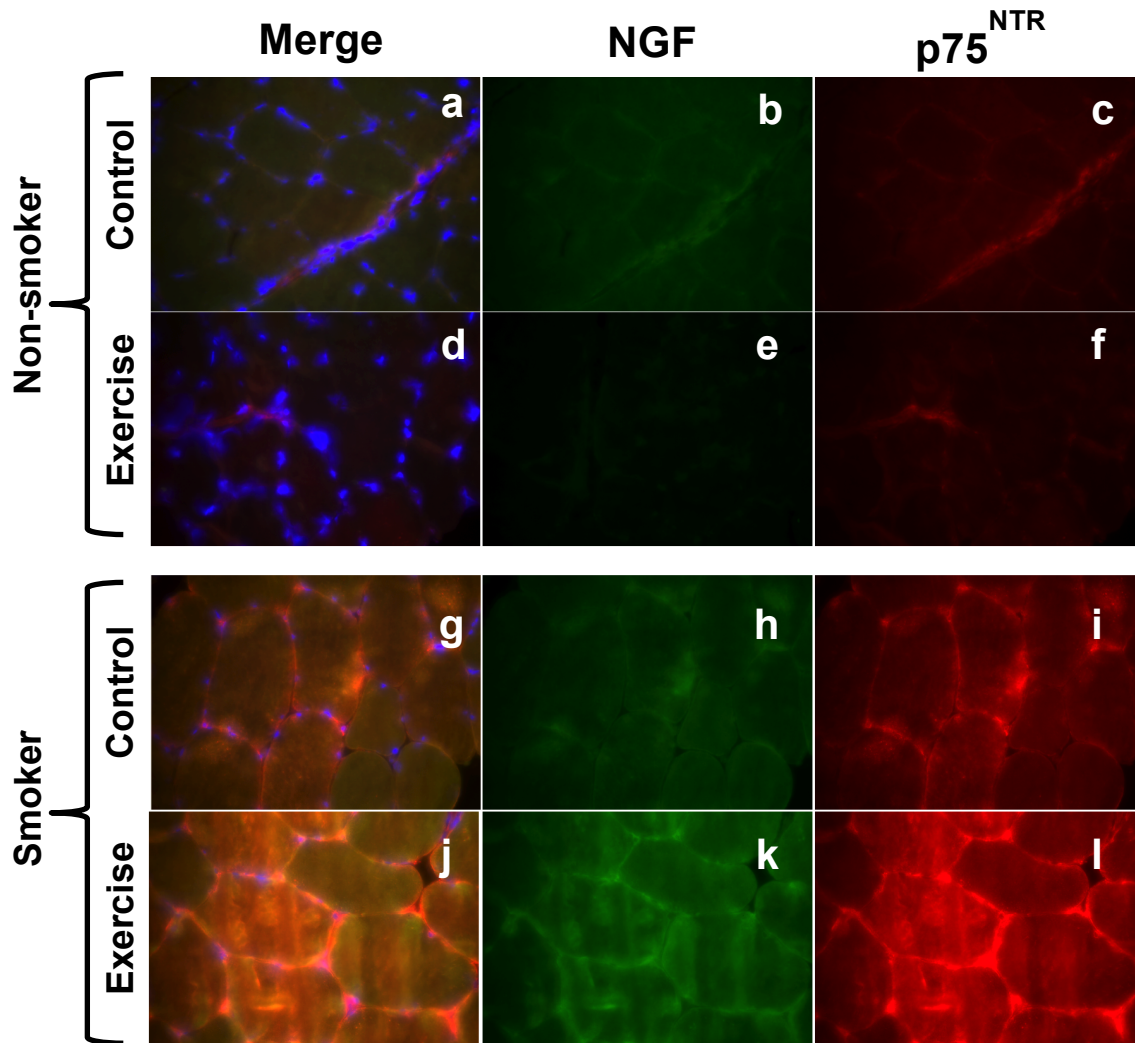
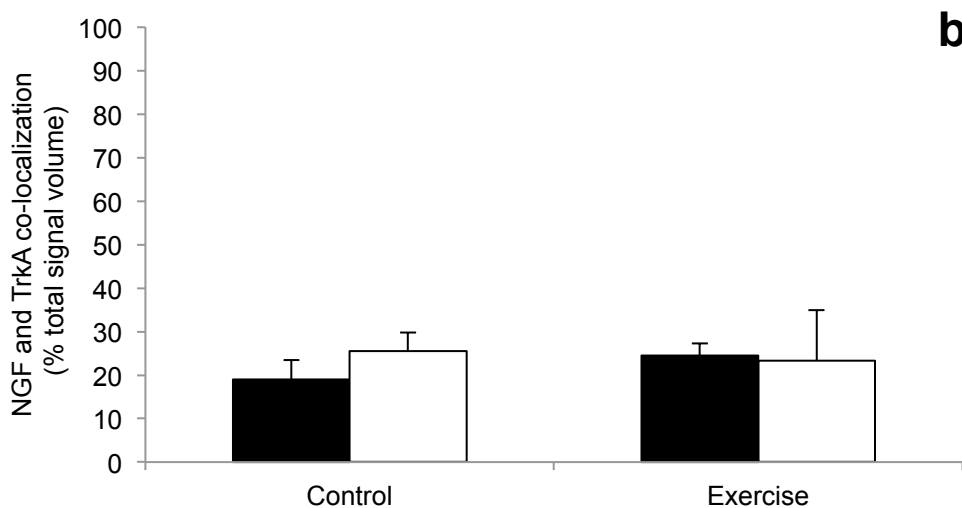
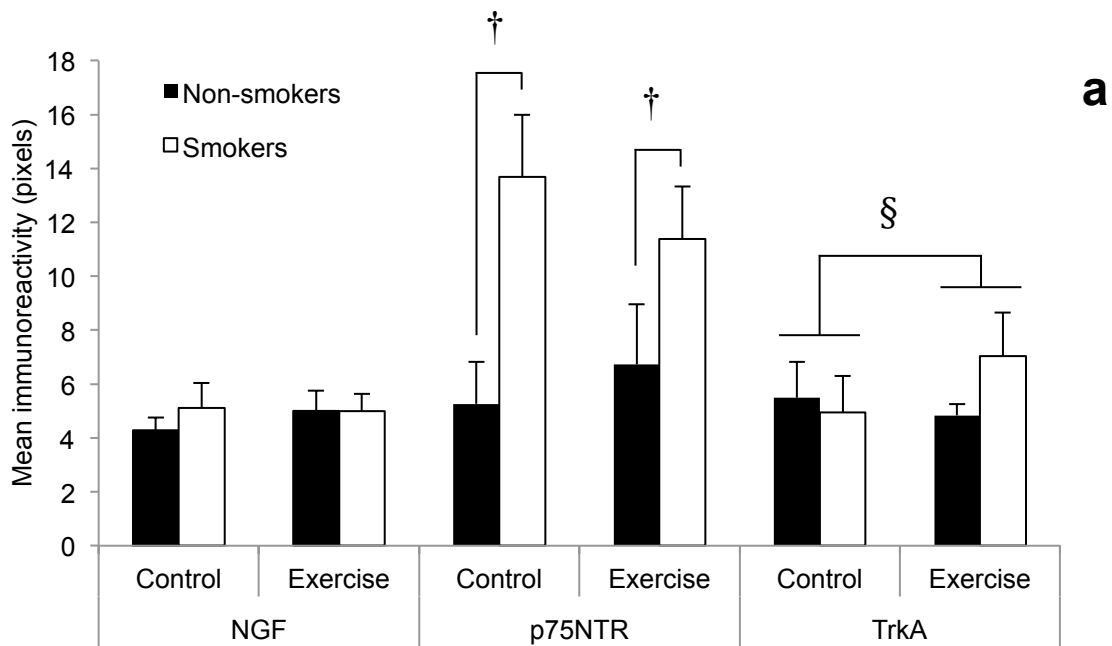
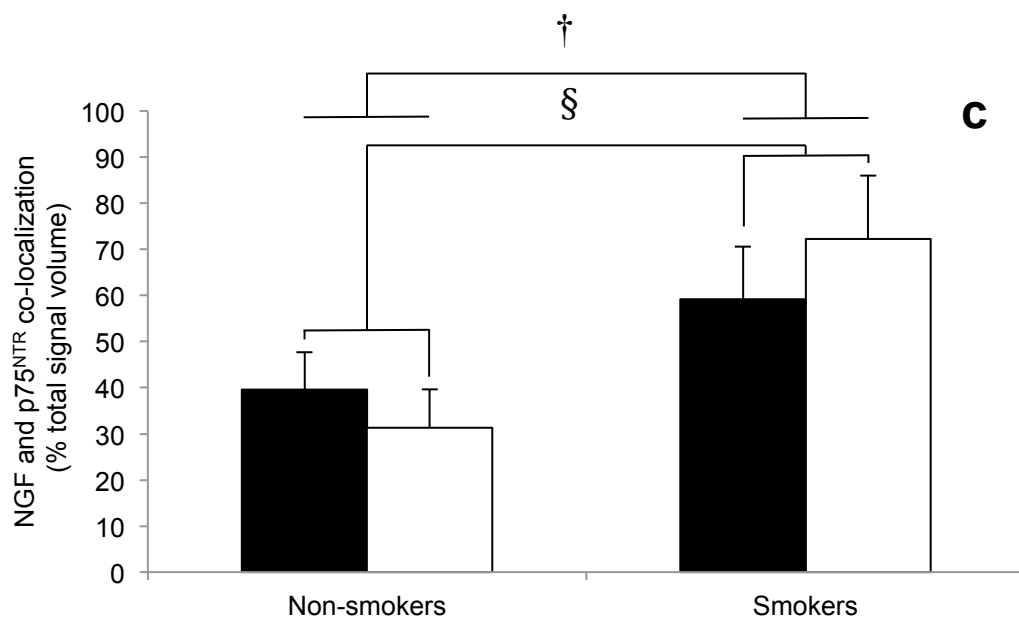


Figure 5.12: NGF, TrkA, and p75^{NTR} Expression and Co-Localization.

Protein expression (a), as mean immunoreactivity, of NGF, TrkA, and p75^{NTR} are shown at 48h after eccentric exercise in non-smokers (■) and smokers (□). Co-localization of NGF with TrkA (b) and NGF with p75^{NTR} (c) are shown in control (closed bars) and exercised (open bars) legs. * p<0.05, effect of exercise; § p<0.05, interaction (group*exercise); † p<0.05, effect of group (smoke). NGF, nerve growth factor; TrkA, tyrosine receptor kinase A; p75^{NTR}, p75 neurotrophin receptor.





CHAPTER VI

SUMMARY

The **primary goal of this dissertation** was to understand how smoking affects muscle damage and recovery. Data primarily from large-scale epidemiological studies indicate that smokers are at greater risk for musculoskeletal injury (5, 145, 157, 182, 286) (145, 157). Further, smokers have a greater risk for complications and prolonged healing time after injury or surgery (82, 117, 163). The mechanisms underlying these phenomena in skeletal muscle damage had not yet been explored, although dysregulated inflammatory responses are potential candidates (17, 307, 312, 316). To investigate muscle injury and recovery in smokers, I used eccentric exercise contractions (ECC), a model of transient muscle damage, to elicit muscle stress.

Prior to this dissertation, the effects of smoking on the response to ECC were unknown. Therefore, the **first goal of this dissertation** was to test if the functional responses of smokers to muscle stress were different from non-smokers. I found that smokers experienced greater fatigue during exercise as well as greater delayed hyperalgesia, in the form of delayed onset muscle soreness (DOMS), after exercise. These novel findings provide insight into not only functional changes that may place smokers at a higher risk for injury when performing fatiguing tasks, but also suggests that there are molecular differences between smokers and non-smokers that may affect their ability to recover from such strain.

Soreness typically peaks in smokers at 2d post-ECC, a stage of flux after ECC during which the inflammatory balance shifts from favoring pro-inflammatory

actions (early) to favor anti-inflammatory ones (late). However, little is known regarding the molecular responses of muscle to ECC at 48h after exercise. Therefore, the **second goal of this dissertation** was to identify differences in the skeletal muscle transcriptome between the early and late inflammatory phases in non-smokers and to use these results to identify candidate genes and pathways that may explain findings (greater soreness, less fatigue resistance) from Dissertation Study 1 (Chapter 3) for further study. Transcriptome analysis indicated that canonical (p65: REL-A, v-rel avian reticuloendotheliosis viral oncogene homolog A) nuclear factor kappa-beta (NFκB), extracellular regulated kinase (ERK)1/2, and nerve growth factor (NGF)-related signaling were all enhanced early and suppressed later. These pathways may act early to promote pro-inflammatory signaling, proliferation, and DOMS. Thus, their reduced signaling activity at the later time point could indicate a return toward inflammatory balance and subsequent DOMS resolution. These pathways are also altered in various non-muscle tissues/cells with smoke exposure, and so were further indicated for study in smokers.

The **final goal of this dissertation** was to test potential mechanisms underlying the greater muscle fatigue found in Dissertation Study 1 (Chapter 3) and to test the pathways identified in Study 2 (Chapter 4) in smokers at 48h after ECC. Smokers had a lower percentage of Type I (oxidative, fatigue resistant) muscle fibers. In addition, activity of the non-canonical NFκB pathway (REL-B, v-rel avian reticuloendotheliosis viral oncogene homolog B), which stimulates oxidative metabolism and was increased in non-smokers, also showed no change for smokers. The suppressed REL-B activity suggests a mechanism through which smokers may

suppress oxidative metabolism and, over time, muscle may adapt by shifting fiber types to favor the less fatigue-resistant Type II fibers. These data support a mechanism through which smoking results in the higher muscle fatigability.

I found that the activity of the canonical (p65) NFκB pathway, suppressed in non-smokers after ECC, was unchanged in smokers. Smokers also had a lower nuclear ERK activity capacity and ERK activity in the cytosol, which could blunt signaling downstream of ERK and limit the overall ERK activity response. However, this difference from non-smokers does not appear to relate to the NFκB signaling, as exercise did not affect ERK activity. Taken together, these data suggest that smokers may have baseline suppression of proliferation and inflammatory signaling downstream of ERK, and an impaired inflammatory response to muscle damage. These effects could attenuate proliferation, undermine inflammatory resolution, and blunt recovery, repair, and adaptation to muscle damage in smokers.

Although there were no differences in overall NGF expression, smokers expressed higher protein levels of the low-affinity NGF receptor, p75^{NTR}. In addition, NGF co-localization to p75^{NTR} was greater in smokers after exercise yet decreased in non-smokers. These data may explain why NFκB activity, which occurs downstream of p75^{NTR}, fails to decrease at 48h after ECC in smokers, and suggests a way in which there is a break down of pro-inflammatory resolution. These data further suggest that smoking affects NGF signaling after muscle damage and provide a mechanism through which smoking increases DOMS.

With this dissertation, I provided evidence that smoking attenuates fatigue resistance and enhances hyperalgesia, and mechanisms through which each of these

gross responses may be affected. These data suggest two mechanisms that may increase heightened risk for muscle injury. Further, understanding the effects of smoking on muscle response to injury may lead to more specific recommendations for fitness interventions in smokers. Finally, these data may lead to the development of strategies to reduce overall risk for injury and promote muscle healing and regeneration.

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