AN ABSTRACT OF THE DISSERTATION OF

Seung Jun Choi for the degree of Doctor of Philosophy in Exercise and Sport Science presented on October 22 2010.

Title: Role of Myosin Heavy Chain Polymorphism in Differential Susceptibility of Muscle Fibers to Eccentric Contraction

Abstract approved: _____

Jeffrey J. Widrick

Relationships between myosin heavy chain (MHC) isoform content and eccentric-induced damage were investigated in chemically skinned muscle fiber segments. Fibers were subjected to an eccentric contraction standardized for strain magnitude (0.25 fiber length) and velocity (0.50 maximal shortening velocity). Fiber MHC isoform content was identified by SDS-PAGE. Pre- to post-eccentric changes in Ca^{2+}-activated specific force, an accepted marker of damage, were analyzed using multiple linear regression

The first study utilized vastus lateralis (VL) fibers from young human subjects (25±2 yrs, N=10). When pre-specific force was held constant, fibers expressing type I or type IIa MHC showed identical post-eccentric force reductions while fibers co-expressing the type IIa and IIx MHC showed significantly greater force deficits.

The limited number of monomorphic (type I, IIa) and polymorphic (type IIa/IIx) fibers in the human VL made it difficult to distinguish the role of MHC co-expression on these results. Therefore, in the second study, we utilized muscle fibers from C57BL/6 mice in order to examine the full range of mammalian MHC monomorphic (type I, IIa, IIx, IIb) and most common polymorphic (type I/IIa, IIa/IIx, IIx/IIb) fibers. Fibers expressing multiple MHC's showed a 3-fold greater post-eccentric force loss than fibers expressing a single MHC isoform. These studies point to an association between fiber MHC polymorphism and increased susceptibility of the myofilament lattice to high mechanical strain.

Finally, we tested VL fibers from elderly subjects (78±2 yrs, N=8) in order to investigate how susceptibility to damage may be altered by age. Comparision to the young subjects of the first study revealed that resistance to eccentric-induced damage was preserved in type I fibers well into the 8th decade of life. Likewise, type IIa/IIx fibers from young and old subjects showed similar susceptibility to damage. However, type IIa fibers from elderly subjects showed a 4-fold greater force reduction after the eccentric treatment. These results indicate a fiber type specific deterioration of the myofilament lattice with age.

The novel findings of this research are the relative resistance of MHC monomorphic fibers and the greater susceptibility of MHC polymorphic fibers to eccentric damage and how these relationships are altered with aging.

Role of Myosin Heavy Chain Polymorphism in Differential Susceptibility of Muscle Fibers to Eccentric Contraction

by

Seung Jun Choi

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APPROVED:

Major Professor, representing Exercise and Sport Science

Chair of the Department of Nutrition and Exercise Sciences

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Seung Jun Choi, Author

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CONTRIBUTIONS OF AUTHORS

Dr. Jeffrey J. Widrick assisted in the design of the studies, performed the surgical procedures on the mice, assisted with data analysis, and helped the write the manuscripts.

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Chapter 1

Introduction

Seung Jun. Choi

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According to the American Academy of Orthopaedic Surgeons (AAOS), there were more than 57.2 million musculoskeletal injuries in 2004, including strains and sprains, in the United States (Hutchinson, 2009). These injuries result in an estimated $127.4 billion in treatment costs, and this cost has risen 37 % since 1996. In addition, each year, one million individuals experience job-related musculoskeletal injuries, resulting in an estimated $50 billion in treatment costs and lost wages (Council, 2001). When combined with injuries sustained during recreational activities, the total economic cost of musculoskeletal injuries comes to more than $200 billion annually. AAOS also reports that about half of musculoskeletal injuries occur in the home during excessive use or sudden movements. Thus musculoskeletal injuries occur in our home life as well as in recreational and occupational situations.

Contraction-induced muscle injuries: One of the primary tissues susceptible to home, recreational, and occupational injuries is skeletal muscle. The National Research Council and the Institute of Medicine (Council, 2001) identified activity-induced muscle injury and dysfunction as a key component of musculoskeletal injuries. Skeletal muscle injury typically occurs following excessive muscular activity, particularly if the muscle is under tension and stretched by an opposing force that is greater than the force generated by the muscle. Under these conditions, muscles can function as brakes to slow limb movement, or shock absorbers to dissipate energy. Everyday examples of activities

that require muscles to function as brakes or shock absorbers include downhill walking or running, lowering a heavy object, and landing from a jump.

A braking or shock absorbing contraction is called an eccentric, pliometric, or lengthening contraction. An eccentric contraction is much more likely to injury a muscle compared to an isometric contraction or a contraction in which the force producing muscle shortens (Faulkner et al., 1993; Allen, 2001; Warren et al., 2001; Lieber & Friden, 2002; Proske & Allen, 2005). General features of eccentric-induced muscle injury are well documented and include disruption of intracellular muscle structure, prolonged muscle weakness and dysfunction, a delayed-onset muscle soreness, and inflammation (Friden et al., 1983; Newham et al., 1983b; Clarkson & Sayers, 1999; Byrne et al., 2004; Proske & Allen, 2005). Several weeks are required for the affected tissue to fully regenerate and recover from eccentric-induced muscle injury (McCully & Faulkner, 1985; Lieber et al., 1994; Lowe et al., 1995). The magnitude of eccentric-induce muscle injury depends upon the number of eccentric contractions, and the strain or magnitude of stretch, but not the rate or velocity of stretch (McCully & Faulkner, 1986; Lieber & Friden, 1993; Warren et al., 1993a; Brooks et al., 1995; Lynch & Faulkner, 1998).

Mechanisms underlying injury: Because of their prevalence and costs, it is important to understand the mechanisms underlying the pathology of muscle injuries. This knowledge may then lead to effective ways of preventing, treating, and rehabilitating injuries. Armstrong et al (Armstrong et al., 1991) proposed that

contraction-induced injury was due to *an initiating event* that targeted a *population of susceptible cells*. The initiating event in eccentric-induced muscle injury is most likely mechanical in nature (Friden & Lieber, 1992; Lieber & Frid, 1999; Friden & Lieber, 2001b). Morgan and colleagues (Morgan, 1990; Morgan & Proske, 2004) propose that sarcomere length heterogeneity leads to a few weaker sarcomeres when muscle is stretched or lengthened. These weak sarcomeres stretch more radially and widely than other stronger sarcomeres. These overstretched sarcomeres lead to structural distortions that are propagated longitudinally and rapidly through the myofibril, leading to physical disruption of the sarcomere, membrane damage and impairment of excitation contraction (EC) coupling (Faulkner et al., 1993; Allen, 2001; Warren et al., 2001; Lieber & Friden, 2002; Proske & Allen, 2005). Muscle stretch may open stretch activated Ca^{2+} channels and Ca^{2+} may enter into the cell via these channels or through ruptures in the sarcolemma (Overgaard et al., 2002). All of this leads to an increase in intracelluar Ca^{2+} levels (Balnave & Allen, 1995; Ingalls et al., 1998), which may activate intracellular proteases, such as calpain (Belcastro, 1993). Leukocyte infiltration (neutrophils, natural killer cells, marcrophage) and increased pro-inflammatory cytokines (interleukin-1β, interleukin-6, tumor necrosis factor-α) are attracted to the injured site and contribute to further degradation of muscle tissue (Peake et al., 2005). Activation of myogenic precursor cells, or satellite cells is required for regeneration of new myofibers and reconstitution of contractile structure (Charge & Rudnicki, 2004).

Population of susceptible cell: In contrast to advances in defining the initiating event, the identification of populations of susceptible cell has lagged, resulting in gap in our understanding of the mechanisms of injury. Muscle is a heterogeneous tissue, containing cells with different contractile properties, metabolic profiles, and fatigue resistance. These fiber types are based upon the myosin heavy chain (MHC) expressed by the fiber. Based on MHC, human muscle fibers can be categorized into three fiber types: type I, IIa, and IIx (Smerdu et al., 1994; Ennion et al., 1995). Type IIa or IIx fibers produce slightly greater force, contract faster, and produce substantially greater power than type I fibers, whereas type I fibers are more fatigue resistant than the other fiber types.

It is generally accepted that fast muscle fibers are more susceptible to eccentric contraction than slow muscle fibers (Friden et al., 1983; Jones et al., 1986; Lieber & Friden, 1988; Warren et al., 1994). This idea is based on histological and functional experiments performed on human and animal models. However, a critical appraisal of this body of work shows that the evidence supporting the idea that fast muscle fibers are more susceptible to eccentric injury is far from complete or conclusive. Histological approaches to differentiate injured or non-injured fiber population is limited due to lack of the sensitivity to identify not only fibers showing functional deficits (Lieber & Friden, 1988), but also fibers co-expressing multiple myosin heavy chain (MHC) isoforms (Pandorf et al. 2010). Functional approaches performed on isolated mouse muscle (soleus and extensor

digitorum longus (EDL)) are limited by fiber type heterogeneity and muscle architectural differences. For instance, mouse soleus muscles are not constituted with only slow fiber types, and mouse EDL muscle express an extra MHC isoform, type IIb, which is not founded in human limb muscle (Smerdu et al., 1994; Ennion et al., 1995). Furthermore, its slow type I MHC isoform has a faster velocity than the human slow type I MHC (Widrick et al., 1997b). Thus these species differences could confound generalization of animal data to humans. Also, the differences of muscle architecture between mouse soleus and EDL muscle resulted in the differentiation of applied strain amount to each muscle, due to differences in the fiber length to muscle length ratios of these muscles (Warren et al., 1994; Consolino & Brooks, 2004).

Aging on eccentric-induced muscle injury: In 2000, 35 million people over the age of 65 lived in the United States, accounting for about 12 % of the total population. The older population is predicted to double by 2030 and will represent about 20 % of the total U.S. population (Gist et al., 2004). In addition, the total number of individuals over the age of 65 with chronic disability is also increasing (Manton & Gu, 2001). Considerable attention has been directed toward identifying the symptoms and mechanisms underlying aging-related muscle dysfunction. Aging is generally associated with a decline in skeletal muscle mass and a gradual deterioration of muscle function, including a loss of force and a slowing of

shortening velocity (Doherty, 2003; Goodpaster et al., 2006; Lynch et al., 2007; Thomas, 2007).

Aged muscles are more sensitive to eccentric contraction, at least in animal models (Brooks & Faulkner, 1988; Faulkner et al., 1990; McBride et al., 1995; Brooks et al., 2001). However, human studies examining the effect of eccentric-induced muscle damage on old adults have not reached a consensus. Several human studies have reported that older adults show an increased susceptibility to damage by a single bout of eccentric exercise performed by knee extensors compared to younger adults (Manfredi et al., 1991; Ploutz-Snyder et al., 2001). In contrast, others have reported that the loss of maximal isometric contraction was the same (Dedrick & Clarkson, 1990) or less for old adults vs. young adults following voluntary eccentric exercise by the elbow flexors (Lavender & Nosaka, 2006a; Lavender & Nosaka, 2006b). These contradicting results of eccentric-induced muscle damage on aging may be due to the inability to adequately control important factors contributing to muscle damage, such as strain magnitude (McCully & Faulkner, 1986), intramuscular fiber pennation, and motor unit recruitment patterns in human muscle. Furthermore, age group differences in muscle mass, fiber type composition and mechanical stress may complicate experimental design and interpretation.

To our knowledge, there are few studies that have measured susceptibility of human muscle fibers after eccentric-contraction, especially in muscles

experiencing sarcopenia. Furthermore, any preferential damage to fast twitch fibers could exacerbate the symptoms of sarcopenia. It is therefore critical to understand how different fiber types respond mechanically to eccentric-induced muscle injury, especially for aged populations.

Novel experimental approaches: Single skinned fiber preparations can avoid limitations of previous studies by allowing the investigator control over strain magnitude and velocity under standard experimental conditions of temperature, ionic strength and activating Ca^{2+} concentration. The issue of fiber type heterogeneity can also be overcome by identification of MHC isoforms in the same of single muscle fiber segments used for the physiological assays. Therefore, the purpose of present study was to investigate the role of MHC isoform expression in differential susceptibility of muscle fibers to eccentric contraction. This relationshop has never been examined with the same rigor that has been applied to other mechanical properties of muscle cells, e.g. the relationship between fiber type and shortening velocity. A maximally activated, chemically permeabilized fiber segment preparation confines damage to the filament lattice, the site where the damage process has been proposed to originate (Morgan, 1990; Morgan & Proske, 2006). This is another adavantage of the preparation.

Eccentric-Induced Muscle Injury

Based on the changes in muscle length, skeletal muscle performs three different modes of contraction while generating force. The first type of contraction is an isometric contraction. It occurs when the muscle produces force without a length change. Cross-bridges are still cycling, but sliding of the filaments does not occur. Pushing against a wall is an example. The second type of contraction is a concentric, miometric, or shortening contraction. It occurs when the muscle shortens while generating force. This type of contraction is primarily responsible for acceleration in movement. Lifting an object is an example of this type of contraction. The third and final type of contraction is an eccentric, pliometric, or lengthening contraction. This type of contraction occurs when active muscles are lengthened by external forces. It is primarily responsible for deceleration in movement. Lowering heavy objects, downhill running, and landing from a jump are examples, and all require skeletal muscle to act as a brake or shock absorber. In summary, the main role of isometric contractions is as a stabilizer of joints. Concentric contractions are mainly performed to produce power for limb rotation and movement, and eccentric contractions mainly act as energy absorbers to slow limb movements. Most physical activities require a combination of isometric, shortening and lengthening contractions (Friden et al., 1983; McCully & Faulkner, 1985).

Among these different types of contractions, eccentric contractions result in greater muscle damage then isometric or shortening contractions (Faulkner et al., 1993; Allen, 2001; Warren et al., 2001; Lieber & Friden, 2002; Proske & Allen, 2005). Typical functional consequences of eccentric contractions are a reduction in peak power, a loss in peak force, a slowing of shortening velocity, and a shift in optimal muscle length to a longer length (Katz, 1939; Jones et al., 1997; Brockett et al., 2001; Morgan et al., 2004; Widrick & Barker, 2006). Thus, it resembles muscle fatigue in several characteristics, such as a decline in force, shortening velocity, and power (Fitts, 1994). The difference between muscle fatigue and damage is whether muscle function is able to recover within a short period of time. In other words, muscle fatigue is a generally short-lived dysfunction, whereas muscle damage is a prolonged dysfunction that needs several days for complete recovery (Hough, 1902).

The iniatating event in eccentric-induced muscle injury is thought to be mechanical in nature (Friden & Lieber, 1992; Lieber & Frid, 1999; Friden & Lieber, 2001a). Evidence suggests that the reduction in isometric force is due to the disruption of single or small groups of sarcomeres (Morgan & Allen, 1999), and/or the failure of some step in the excitation-contraction coupling pathway (Warren et al., 2001). After the initial event, symptoms of delayed onset of muscle soreness (DOMS), muscle stiffness, and muscle swelling appear (Jones et al., 1986; Stauber, 1989; Armstrong et al., 1991; Kendall & Eston, 2002). The magnitude of eccentric-

induce muscle injury depends upon the number of eccentric contractions, the strain or magnitude of stretch, but not the rate or velocity of stretch (McCully & Faulkner, 1986; Lieber & Friden, 1993; Warren et al., 1993a; Brooks et al., 1995; Lynch & Faulkner, 1998). Even though these initial mechanisms and symptoms are generally accepted, many details, such as the identification of populations of susceptible cell has lagged, resulting in gab in our understanding of the mechanism of injury.

Excitation-Contraction Coupling

Excitation-contraction (EC) coupling is the term to describe the physiological pathway by which the depolarization of the sarcolemma is linked to the sliding of cross-bridges, resulting in muscle contraction. The EC coupling pathway begins with the release of acetylcholine by the alpha-motor neuron into the neuromuscular junction, resulting in the generation of an action potential. The action potential is propagated along the sarcolemma and into the transverse tubules (t-tubule), which are invaginations of the sarcolemma. Depolarization of the t-tubules causes calcium ion (Ca^{2+}) release from the sarcoplasmic reticulum (SR). The precise mechanism, by which the depolarization of the t-tubules causes the release of Ca^{2+} is not clear. It is generally accepted that the action potential in t-tubules activates the dihydropyridine receptor (DHPR), also called the voltage sensor or the charge sensor, which is mechanically linked to the ryanodine receptor

at the triad junction. Depolarization results in a conformational change of the DHPR (Chandler et al., 1976). The ryanodine receptor (RyR) is a Ca^{2+} release channel. There are two different methods by which Ca^{2+} is released through RyR; depolarization induced calcium release (DICR), and calcium induced calcium release (CICR). Because some RyR are mechanically connected to DHPR, it thought they are opened by a conformation change of the DHPR. This conformational change is transmitted to DICR channel via a linkage, and "opening or unplugging" of the DICR channel, initiating Ca^{2+} release from the SR. The released Ca^{2+} may act to stimulate CICR from uncoupled RyR's. Ca^{2+} binds to the troponin C, initiating cross-bridge cycling.

Cross-bridge Cycle

The cross-bridge theory, proposed by Andrew Huxley in 1957 (Huxley, 1957) states that the sliding of myofilaments occur via a cyclical attachment and detachment of myosin heads to actin without any change in filament length. The cross-bridge cycle (Lymn & Taylor, 1971) combines the hydrolysis of adenosine triphosphate (ATP) with this cross-bridge theory. That is, a sequence of enzymatic reactions are coupled to the binding and splitting of ATP, and release of the hydrolysis products, such as inorganic phosphate (Pi), and adenosine diphosphate (ADP), to create the power stroke necessary to slide the filaments. Also, Ca^{2+} released from the SR binds to troponin C on the thin filaments, causing myosin (M)

heads of thick filament to strongly bind with actin (A) to form a cross-bridge by exposure of the actin active site.

The general cross-bridge cycles is as follows; 1) actin and myosin are bound in a rigor actomyosin complex; (A•M) 2) ATP binds to the A•M complex and causes rapid detachment of the actin molecule; (A+M•ATP) 3) hydrolysis of ATP brings about a conformational change in the myosin, and the myosin lever arm is repositioned at a pre-power stoke position; (A+M•ADP•Pi) 4) The M•ADP•Pi complex reattaches to action; (A•M•ADP•Pi) 5) the dissociation of Pi is associated with a transformation from a weakly bound, low force generating complex to a strong bound, high-force generating complex; (A•M'•ADP) 6) ADP is released, returning to the rigor actomyosin complex; (A•M). During muscle contraction, actin and myosin molecules continually undergo this cycle of detachment and re-attachment. In detail, when the muscle contracts isometrically, the filaments are stationary, thus the cross-bridges detach from the same position where they attach, with about 80% of cross-bridges formed at any instant. During the concentric contraction the filaments slide over the myosin filament and pull the Z discs toward the center of the sarcomere. At 25% of maximal shortening velocity, about 50% of cross-bridges are formed. During the eccentric contraction, the filaments still pull the Z disc in the direction of the center of the sarcomere, but the filament actually slide in the opposite direction due to an overwhelming force stretching the muscle.

Structural change within the myosin head while it is attached to actin is thought to be the direct cause of the filament's sliding, pulling actin toward the center of the sarcomere. During a maximal isometric contraction, the strongly bound state are thought to be the dominant form, whereas during isotonic shortening contraction , skeletal muscle myosin spends only 5% of the cross-bridge cycle time in the strongly bound state (Sweeney & Houdusse, 2004). The transition rate from weak binding state to strong binding state is thought to limit the peak force development, whereas the maximum shortening velocity (V_o) is highly correlated with the actomyosin ATP hydrolysis rate. Mechanically, V_o is limited by the ADP release step, because it is the slowest step in cross-bridge cycle (Siemankowski et al., 1985).

Mechanisms of Eccentric-induced muscle injury

Possible mechanisms responsible for eccentric-induced muscle injury are activation impairment and structural disruption of the sarcomere. Theses two factors seem to be the main sources of eccentric-induced muscle injury. Rather than being separate mechanisms they may be complimentary and interact with each other.

Activation Impairment

The first possible mechanism is a failure of Ca^{2+} release and uptake. Warren and associates proposed that impaired EC coupling plays a major role in eccentric-

induced muscle injury (Warren et al., 1993c; Warren et al., 1994; Ingalls et al., 1998).

The failure of EC coupling has been demonstrated after eccentric exercise (Balnave & Allen, 1995), and it suggests that less calcium is released per action potential. Caffeine bypasses the normal EC coupling process and directly opens the RyR, resulting in release of Ca^{2+} from SR (Endo, 1977; Martonosi, 1984). Warren hypothesized that if the reduced force after eccentric contractions can be bypassed by caffeine, then the reduced force could be attributed to EC coupling failure. To test this hypothesis, the mouse soleus muscle was exposed to bicarbonate buffer containing caffeine after 20 eccentric contractions (Warren et al., 1993c). The caffeine-elicited isometric force in injured mouse muscles was not different from the control muscle, even though maximal isometric tetanic force of injured muscle was reduced 43%. Accordingly, Warren and colleagues proposed an impaired EC coupling system as a main source of dysfunction.

By using a skinned single fiber preparation, the EC coupling pathway can be bypassed and the fiber activated by directly application of saturating Ca^{2+}. Thus, under this condition, the reduced maximal Ca^{2+}-activated force can be attributed to the disruption or alteration of the force-generating and transmitting structures. Thus, Warren et al (Warren et al., 1994) tested the force reduction in the isolated EDL muscle after 15 eccentric contractions, and also measured the maximal Ca^{2+}-activated force on skinned muscle fiber from that isolated EDL muscle. The result

showed that the maximal isometric force of the isolated muscle was reduced by 69%, whereas the maximal Ca^{2+}-activated force was reduced by 34%. Therefore, they concluded that the physical disruption of muscle ultrastructure may explain about half of the strength loss of the muscle.

Balnave and Allen (Balnave & Allen, 1995) directly measured Ca^{2+} release. They conclude that Ca^{2+}-activated force was not significantly affected after 10 eccentric contractions at 25% strain on living muscle fibers. However, with 50% strain, the about 79% of the force reduction can be attributed to the disruption of the sarcomere.

To investigate how long the EC coupling failure contributes to the force deficit, measurements were made at 0, 1, 3, 5, and 14 days after in vivo 150 eccentric contractions. The study concluded that during the first 5 days after injury, EC coupling failure is thought to account for about 57~75% of the strength deficit. The EC coupling failure is diminishing by 5 days after and is resolved by 14 days after the injury (Ingalls et al., 1998). Thus, the disrupted muscle ultrastructure is thought to account for about 25~43% of the strength deficit.

The possible failure sites in the EC coupling pathway are, the sarcolemma, the t-tubule, the DHPR, the linkage between the DHPR and the RyR, the RyR, and the SR. First, at the sarcolemma, EMG amplitude was reduced 9% after eccentric contractions (Warren et al., 1999), suggesting a failure in action potential propagation. However, because force was reduced 47%, action potential failure is

not considered a major contributor to dysfunction. Second, the ability of the t-tubule to conduct action potential and the intrinsic properties of the DHPR do not appear to be impaired significantly. Because the potassium (K^+) induced force was reduced proportionally with maximal isometric force after 150 in vivo eccentric contractions, and it suggests the failure site of EC coupling process is below the level of the DHPR (Ingalls et al., 1998) since K^+ trigger muscle contraction by depolarization of the t-tubule and voltage sensor (Dulhunty, 1991). More recently the t-tubules have been observed to be broken after 40% of strain, and it may due to movement, originating from the heterogeneity of sarcomere length (Yeung et al., 2002). Third, the linkage between the DHPR and the RyR is though to be fragile. Warren (Warren et al., 2001) proposed that the EC coupling failure result from the weak mechanical link between DHPR and RyR, because the conformational change of DHPR may be failed to be communicated to the RyR. Lastly, at the SR, the intrinsic function of ryanodine receptor decreases by 16% over the first 3 days after in vivo eccentric injury (Ingalls et al., 2004), but this progressive worsening of SR function is not associated with a further impairment in muscle functions, because the rates of Ca^{2+} release and uptake by the SR are not significantly impaired (Warren et al., 2001).

Cross-Bridge Impairment

It is hard to conclude that all of the muscle dysfunction induced by eccentric contraction is caused only by EC coupling failure. As mentioned above, Balnave and Allen (Balnave & Allen, 1995) founded evidence for both the EC coupling impairment and the structural disruption with 25% and 50% of strain, respectively. Also, some find that the force deficit can not be overcome by caffeine treatment in frog fibers (Morgan et al., 1996), and toad muscle (Allen, 2001). Finally, impaired EC coupling system can not readily explain two significant characteristic features associated with damage from eccentric contraction: a shift in the length-tension relationship of the muscle and increased passive tension.

An alternative possible mechanism for eccentric-induced muscle dysfunction is based on the heterogeneity of sarcomere length and the length-tension relationship. This is called the “popping sarcomere hypothesis” proposed by Morgan (Morgan, 1990; Morgan & Proske, 2004). It is derived from two characteristics of muscle contraction: the produced force by eccentric contraction is greater than the force produced by concentric contraction (Katz, 1939) and the presence of unstable sarcomeres lengthened onto the descending limb of their length tension relationship. The decending limb of the length tension relationship refers to a linear decrease in active tension as the sarcomere length is lengthen beyond optimal length, and it is correlated with decreasing overlap of thick and thin filaments (Gordon et al., 1966).

The popping sarcomere hypothesis assumes the presence of irregular sarcomere lengths. This results in eccentric-induced muscle injury because the longest sarcomeres become the weakest sarcomere when stretched out to the descending limb of their length-tension relationship. Thus, these longer, weaker sarcomeres are stretched more rapidly and widely than other sarcomeres. As a consequence, weaker sarcomeres take up most of the stretch until its total tension is balanced by the tension produced by in series sarcomeres, which are on the plateau of their length tension relationship. The thin and thick filaments making up this overstretched sarcomere may not re-interdigitate when the muscle relaxes (Talbot & Morgan, 1996). If this occurs continually, more and more sarcomeres will become overstretched and dysfunctioned. Once one or more sarcomere has become disrupted, the damage may expand longitudinally to adjacent sarcomeres in the myofibril and transversely to adjacent myofibrils. The longitudinal and transverse propagation of damage may also impact membranes of the sarcoplasmic reticulum, tranverse tubules, and sarcolemma (Morgan, 1990). This could then initiate an uncontrolled movement of Ca^{2+} into the sarcoplasm (Yeung & Allen, 2004), triggering the next stage in the damage process. According to this hypothesis, the disruption of muscle ultrastructure precedes EC coupling failure, and it is proposed as a main factor leading to reduced functional properties after eccentric contractions (Proske & Allen, 2005).

One consequence of disrupted sarcomeres is a shift in optimal length to longer lengths (Katz, 1939; Jones et al., 1997; Brockett et al., 2001; Morgan et al., 2004). This shift in optimal length is a reliable and useful measure of the amount of eccentric-induced damage (Talbot & Morgan, 1998). It is proposed that this shift in muscle optimal length after the eccentric contractions is due to the disrupted sarcomeres, which lie scattered at random along the myofibril. The presence of these disrupted sarcoemre in parallel with undisrupted sarcomeres increases the series compliance of the fiber, and the increased series compliance leads to a shift in the muscle's optimal length for peak active force toward longer muscle lengths (Morgan, 1990).

Another consequence of the disruption of sarcomere is the rise in passive tension immediately after eccentric contraction observed in isolated muscle (Whitehead et al., 2001; Whitehead et al., 2003) and single fibers (Joumaa et al., 2007). It is greatest in the region of the muscle's optimal length (Whitehead et al., 2003). The possible mechanism of this rising passive tension is that uncontrolled Ca^{2+} released into the sarcoplasm by eccentric contractions may result in the activation the contractile filaments to develop an injury contracture, thus it causes rising passive tension (Whitehead et al., 2003). In addition, the uncontrolled release of Ca^{2+} into the sarcoplasm may bind with titin, raising the passive tension. The PEVK domain of titin binds Ca^{2+} with high affinity, resulting in an increase in

passive tension of the PEVK domain(Tatsumi et al., 2001; Labeit et al., 2003), which is the main contributor to passive tension of titin (Horowits, 1999).

Sarcomere Heterogeneity

Recently, sarcomere heterogeneity was proposed as a causal relationship between sarcomere strain and fiber bundle injury by eccentric contractions (Patel et al., 2004). This study found that heterogeneity of sarcomeres was increased as the activated muscle bundles underwent successive stretches, and it found a high correlation (r^2=.87) between sarcomere strain and the relative force deficit. Furthermore, the progressive increase in heterogeneity of sarcomere length is inversely related to maximal eccentric force with a correlation coefficient ranging from 0.61 to 0.93. Because the weak sarcomeres are beyond their optimal length, and become weaker or longer as heterogeneity increases, this results in a lower maximal eccentric force. This group also concluded that the contribution of EC coupling to eccentric-induced muscle dysfunction is relatively small, especially as strain increases. Accordingly, the high correlation between sarcomere heterogeneity, and force deficit and maximal eccentric force agree with the popping sarcomere hypothesis.

Summary of Possible Mechanisms

In summary, the initial sequence of events underlying eccentric muscle dysfunction begins with the disruption of a sub-population of sarcomeres due to the heterogeneity of sarcomere length. Then, structural distortions triggered by the disruptions of these sarcomeres leads to membrane damage, which interferes with EC coupling. At the same time, the accompanying stress applied to the sarcolemma leads to opening of cation channels and the rupture of the sarcolemma. All of this produces an increase in intracellular Ca^{2+} levels which triggers proteolysis and fiber breakdown. A key point in that the process is initiated at the level of the myofilament lattice of the sarcomere.

Distinct susceptibility on MHC Isoform

Characteristics of Fiber Types

Based on myosin isoform content, human muscle fibers can be categorized into three fiber types: type I, IIa, and IIx (Smerdu et al., 1994; Ennion et al., 1995). These isoform can also be co-expressed, resulting in hybrid fiber types: type I/IIa, and type IIa/IIx or even type I/IIa/IIx (Pette & Staron, 2001; Caiozzo et al., 2003). Typical characteristics of fiber types are force production, and shortening velocity. Type IIa or IIx fiber produce higher force and contract faster than type I fibers, whereas type I fibers appear to be more fatigue resistant than other fiber types.

Type IIx fiber contract and relax slightly faster than type IIa fibers, but both are faster than type I fiber.

The difference in force production between type I and type IIa or IIx is due to differences in the number of myofibrils in the fiber. All myosin heavy chain (MHC) isoforms seem to produce the same force (Tipton & American College of Sports Medicine., 2006) but type IIa or IIx fibers have a higher density of myofibrils than type I fibers. This is due to the number of mitochondria, because type IIa or IIx fiber have about twofold to threefold less mitochondria than type I fiber, which allows more space for myofibrils (Tikunov et al., 2001). However, the higher concentration of mitochondria makes human type I fiber the most oxidative, and therefore most fatigue resistant, of any fiber type.

Unlike force production, shortening velocity between type I and type IIa or IIx is due primarily to the existence of different MHC isoforms. Each MHC isoform shows a different rate of cross-bridge cycling, and shortening velocity is dependent on this rate. In addition, shortening velocity is also depend upon the myosin light chain (MLC) isoform, but MLC's only modulate shortening velocity (Bottinelli, 2001).

Another characteristic between fiber types is Ca^{2+} sensitivity. Type I fibers have a higher sensitivity to Ca^{2+} than type II fibers, because of the different isoforms of the troponin C subunit (Veigel et al., 1999). In detail, type I fiber has three cation binding site on troponin C subunit: two high-affinity site, which always

occupied by Mg^{2+} or Ca^{2+}, and one low-affinity site, which bind Ca^{2+} specifically, whereas type IIa or IIx fibers have four cation binding site: two high-affinity site for Mg^{2+} or Ca^{2+}, and two low-affinity site for only Ca^{2+} (Gordon et al., 2000). In other words, two Ca^{2+} molecules are required to activate a type II fiber, but only one Ca^{2+} molecule is required to activate a type I fiber. Therefore, at sub-maximal Ca^{2+} concentrations type I fibers produce a higher percentage of their peak force than type II fiber.

Different Susceptibility of Fibers

Studies on humans have reported a greater susceptibility of fast-twitch fibers to damage (Friden et al., 1983; Jones et al., 1986; Asp et al., 1998). However, these studies have used indirect methods to quantify the muscle injury such as histological markers of damage (Friden et al., 1983) or the plasma creatine kinase (CK) concentration (Jones et al.). Plasma CK concentration is frequently used as an index of the muscle damage after eccentric contraction. However, no significant correlation was observed between CK concentration and muscle strength (Friden & Lieber, 2001b). Another study investigated muscle glycogen content after eccentric exercise, and it revealed type II fibers were predominantly recruited (Asp et al., 1998). Thus the authors concluded a selective effect of eccentric contractions on type II fiber but it could not be readily accepted that the glycogen content represents the susceptibility of muscle structure and functional to damage.

Functional approaches performed on isolated mouse muscle (soleus and extensor digitorum longus (EDL)) are limited by fiber type heterogeneity and muscle architectural differences. For instance, mouse soleus muscles are not constituted with only slow fiber types, and mouse EDL muscle express an extra MHC isoform, type IIb, which is not founded in human limb muscle (Smerdu et al., 1994; Ennion et al., 1995). Furthermore, its slow type I MHC isoform has a faster velocity than the human slow type I MHC (Widrick et al., 1997b). Thus, these species differences could confound generalization of animal data to humans. Also, the differences of muscle architecture between mouse soleus and EDL muscle resulted in the differentiation of applied strain amount to each muscle, due to differences in the fiber length to muscle length ratios of these muscles (Warren et al., 1994; Consolino & Brooks, 2004).

Studies using single fibers, obtained from animals report that muscles comprised of predominantly fast fibers are preferentially damaged (Lieber & Friden, 1988; Friden & Lieber, 1992; Macpherson et al., 1996; Vijayan et al., 2001; Brockett et al., 2002; Rader et al., 2007; Lynch et al., 2008). Only three of these studies have directly examined muscle fiber function: two studies examined fibers from the predominately slow soleus and the predominately fast EDL of the rat (Macpherson et al., 1996; Lynch et al., 2008), and another study examined fibers from normal and congenitally clefted goat palates (Rader et al., 2007). Most of these studies have been performed using muscles from rodents. As discussed

above, small mammals and humans do not express all of the same MHC isoforms. Sarcomere heterogeneity after eccentric contractions was also measured with fiber type identification by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Patel et al., 2004). However, they used the pooled fiber bundles from the frog tibialis anterior muscle, which is comprised of two equal part of fastest amphibian muscle fiber type (Lutz et al., 1998). Brockett (Brockett et al., 2002) also studied the vulnerability of different motor units that consisted of mixed fiber types using the cat muscle without identifying the fiber types. None of these studies examined MHC isoform content and function in the same fibers, making it difficult to draw conclusions about damage to individual fiber types.

Even though there is no conclusive mechanism yet, several potential mechanisms were proposed to explain the greater vulnerability of fast-twitch fibers. One proposed mechanism was that fast-twitch fibers fatigued faster than slow-twitch fiber, and the fast-twitch fibers stay in rigor binding stage during cross-bridge cycle due to impaired ATP regeneration. This results in mechanical damage as these rigor fibers aere stretched (Friden & Lieber, 1992; Lieber & Frid, 1999). However, this hypothesis does not hold up because studies report that fatigued muscle is less susceptible to damage or fatigue has no effect (Friden & Lieber, 1992; Mair et al., 1996; Morgan et al., 2004; Choi & Widrick, 2009) and other studies support the fatigued muscle is more resistible to eccentric-induced muscle injury (McCully & Faulkner, 1986; Nosaka & Clarkson, 1997). It has been

suggested that the ultrastructural differences between type I and type II fibers explain the greater vulnerability of fast-twitch fibers to eccentric-induced muscle damage. Fast-twitch fibers have narrower Z-lines compared to slow-twitch fibers (Friden et al., 1983), which reflect fewer attachments for thick and thin filaments (Yamaguchi et al., 1985). Type II fibers contain smaller isoforms of the sarcomeric proteins myomesion and nebulin, which play a role in sarcomere assembly (Agarkova et al., 2004; Prado et al., 2005). Therefore, relatively greater stress is applied to the cytoskeleton of fast fiber, resulting in a higher susceptibility to damage. This phenomenon also confirmed from the reloaded human soleus fiber after 17 days of bed rest and spaceflight (Widrick et al., 1997a; Widrick et al., 1999). He found that the fibers, have weak connections between sarcomeres were relatively weaker than fibers, have normal connection between sarcomeres, due to greater workload. Also, fast fibers express a lower molecular weight, and less elastic, titin isoform than slow fibers (Prado et al., 2005) The less compliant titin in fast fibers may transmit greater stress during eccentric contractions (Horowits, 1992).

Accordingly, it is widely accepted that fast-twitch, or type II fibers tend to be more vulnerable to eccentric-induced muscle injury (Proske & Morgan, 2001; Byrne et al., 2004). However, none of above studies has investigated the relationship between fiber MHC isoform content and its susceptibility to eccentric

contraction using single fiber preparation, isolated from human muscle, with the identification of fiber type.

Although most studies show greater susceptibility of fast-twitch fibers, a few studies report that slow-twitch, or type I fibers, were selectively damaged during eccentric contractions. Armstrong (Armstrong et al., 1983) showed that the deeply embedded slow-twitch fibers were predominantly damaged after downhill running compare to level running. However, downhill running may preferentially recruit the slow fiber type, and this may obscure which type is actually more susceptible. Another study indicated the increased fragment rate in slow-twitch skeletal MHC through magnetic resonance imaging (Mair et al., 1992), and plasma creatine kinase (CK) concentration, which is not a highly correlated index of muscle damage.

In conclusion, while most studies conclude that type II fibers are more susceptible to eccentric-induced muscle injury, these finding are based on indirect measures of damage, conducted on muscles instead of fibers, and if conducted on fibers, have not confirmed MHC isoform context by gel electrophoresis which provide a more accurate determination of the fiber types including hybrid fiber (Glaser et al. 2009; Pandorf et al. 2010). Since most muscles are heterogeneous in the fiber type composition, whole muscle or motor unit studies can not effectively evaluate changes of function occurring in type I and type II fibers following eccentric contractions. Accordingly, it is clear that more data examining the

relationship between fiber type expression and susceptibility after eccentric contraction are required. It will be useful to get additional information of the depth of the cross-bridge change after eccentric-induced muscle injury.

Aging and Eccentric-Induced Muscle Injury

Aging and Functional Changes in Skeletal Muscle

'Sarcopenia' is a widely used term to describe the progressive loss of muscle mass with aging. It is characterized by the loss of skeletal muscle mass, and also by the gradual decline in muscle functional properties, such as a decrease in force generating capacity, maximum shortening velocity, and a general slowing of contraction (Doherty, 2003; Goodpaster et al., 2006; Lynch et al., 2007; Thomas, 2007). This age-related muscle atrophy is thought to be related to a decrease in both muscle quantity (mass), and a decrease in muscle quality (force per cross-sectional area of muscle, proportion of fiber types and metabolic characteristics) (Lynch et al., 2007).

The loss of muscle quantity is thought to be mainly due to a decrease in contractile protein, resulting from both the loss of number of individual muscle fibers and a decrease in the size of remaining muscle fibers. For example, the cross-sectional area of muscle decreased about 25~30%, and muscle strength decreased about 30~40% between ages of 65 to 75 (Porter et al., 1995). This age-related the loss of contractile protein content is likely explained by different rates of protein degradation and protein synthesis (Yarasheski, 2003). The main determinant of the loss of contractile protein content is protein degradation rather than protein

synthesis (Kimball et al., 2004). Also type II fibers are primarily atrophied compared to the type I fibers (Sato et al., 1984; Lexell et al., 1988).

The age-related decline in muscle quality indicates that both the time taken to reach peak twitch tension and the time taken for the muscle twitch response to relax are increased in old muscle (Schertzer et al., 2005). This could be due to several different mechanisms, including an increase in the level of intramuscular collagen and fat, a decline in specific force, an alteration of EC coupling (Delbono et al., 1995; Hook et al., 2001; Plant & Lynch, 2002), neurogenic factors, or motor unit remodeling (denervation and reinnervation).

Even though, it is generally accepted that the contractile properties of muscle were decreased with aging, the effects of aging on the intrinsic ability of single fibers is still equivocal. That is, the results of skinned single fiber studies on maximal isometric force (P_o), and unloaded shortening velocity between young and old human are not consistent.

Several studies have reported that there were significant reduction in the intrinsic contractile properties with aging, such as reductions in maximal isometric force (P_o), normalized force by CSA (P_o/CSA), and unloaded shortening velocity (V_o) (Larsson et al., 1997; Frontera et al., 2000; Krivickas et al., 2001; D'Antona et al., 2003; Ochala et al., 2007). It suggests that both size and quality of each individual muscle fiber were decreased with age. The proposed mechanisms to explain the declined intrinsic force generating ability of aged fibers can be due to

either a lower number of strongly bound cross-bridges during maximal activation or a reduced force-generating ability of each cross-bridge (Ochala et al., 2007).

According to Lowe and colleagues (Lowe et al., 2001), when a muscle fiber contracts maximally, about 32% of myosin heads are in the strong-binding state. However, only 22% of myosin heads are in that state in fibers from older rats. Thus, the decreased P_o in the elderly may due to a decreased numbers of cross-bridges per CSA (D'Antona et al., 2003). Also, there is a loss of myofibillar protein in old rats, which may lead to a reduction of in the motor proteins actin and myosin (Ansved & Larsson, 1990). Slowing of shortening velocity with aging is thought to involve a slowing of the steps within the cross-bridge cycle, such as the actin-myosin cross-bridge detachment rate (Larsson et al., 1997). It is proposed that this occurs without a change in isoform type (Bottinelli, 2001; Canepari et al., 2005), and may be related to glycation of myosin (Ramamurthy et al., 2001).

On the other hand, more recent studies have found that contractile quality of single muscle fiber is maintained during aging (Frontera et al., 2003; Trappe et al., 2003; Korhonen et al., 2006; Slivka et al., 2008) or increased in maximal force in both fiber types with aging (Frontera et al., 2008; Raue et al., 2009). In other words, there was no change of intrinsic ability of single fiber with aging among young and old men and women. It suggests that the intrinsic properties of cross-bridge mechanics are preserved with age (Trappe et al., 2003).

Age-Related Susceptibility to Eccentric Contraction

Several studies have considered the different susceptibility of muscles from young and old to muscle damage induced by eccentric contractions using both animal and human models. The general consensus of the animal studies is that an increased susceptibility of aged muscle to eccentric contractions has been observed consistently in studies performed on isolated rodent muscles (Brooks & Faulkner, 1988; Faulkner et al., 1990; McBride et al., 1995; Brooks et al., 2001). In detail, force deficits were two-fold greater after maximally activated single stretches in single fibers from old rodent EDL muscles, compared with young (Brooks & Faulkner, 1996; Lynch et al., 2008). After a single eccentric contraction (5%, 10%, 20%, or 30% strains), EDL fibers from old rats showed greater force deficit than fibers from young rats up to 20% strain. At 30% of strain, the force deficit was not different between age groups. A breakage rate was reported while fibers were stretched, and it was much greater for old fibers at all strains: about 22% higher at a 20% strain and 45% higher at a 30% strain. Also, the relationship between force deficit and amount of strain was investigated. It was revealed that the force deficit of old muscles had a different pattern compared with young muscle. The young muscles tend to have a linear increase in the force deficit as the strain increased from 20% to 60% (Consolino & Brooks, 2004), whereas the old muscles tended to have curvilinear relationship (hyperbolic curve) between force deficit and strain (Lynch et al., 2008). These characteristics of eccentric contractions have been

exclusively observed in the studies performed on rodent EDL muscle or fibers obtained from EDL muscle (Zerba et al., 1990; Brooks & Faulkner, 1996; Lynch et al., 2008). However, the prodominent MHC isoform in the mouse EDL is type IIb (Danieli-Betto et al., 2005), which is absent form of the limb muscles of larger mammals, such as humans (Smerdu et al., 1994; Ennion et al., 1995). Therefore, not only these functional approaches have limited by fiber type heterogeneity, but also these species differences could confound generalization of animal data to humans.

Human studies examining the effect of eccentric-induced muscle damage and age do not show consistent results. The increased susceptibility of aged muscle to eccentric contraction was reported (Dedrick & Clarkson, 1990; Manfredi et al., 1991), and it contributed to the smaller muscle mass and lower VO_2 max (Manfredi et al., 1991). In contrast, others have reported that the loss of maximal isometric contraction was less for old adults than young adults following voluntary eccentric exercise of the elbow flexors (Lavender & Nosaka, 2006a; Lavender & Nosaka, 2006b), and it contributed to the less degree of muscle damage in older adults due to less mechanical stress during eccentric contraction. These contradicting results of eccentric-induced muscle damage on aging may be due to the inability to adequately control important factors contributing to muscle damage, such as strain magnitude (McCully & Faulkner, 1986), intramuscular fiber pennation, and motor unit recruitment patterns in human muscle. Furthermore, age group differences in

muscle mass, fiber type composition and mechanical stress may complicate experimental design and interpretation.

The increased susceptibility of aged muscle is critical clinical importance because recovery after eccentric damage is slowed (Faulkner et al., 1990; McArdle et al., 2004) or absent (Brooks & Faulkner, 1990; Rader & Faulkner, 2006a, b) in muscles of old rodents. In detail, the contractile functions of damaged old muscle did not recover for up to 2 months (Faulkner et al., 1990; McArdle et al., 2004) or it could bring about permanent loss of muscle mass and force (Brooks & Faulkner, 1990; Rader & Faulkner, 2006a, b). This lack of recovery is thought to be attributed to impaired muscle regeneration (Brooks & Faulkner, 1990; Rader & Faulkner, 2006b, a), resulting from the reduced number of satellite cells, shortened telomeres, and replicate senescence (Gibson & Schultz, 1983; Renault et al., 2002), and the preferential loss of type II fiber (Larsson et al., 1979). Because single muscle fiber preparations can avoid these limitations by allowing the investigator rigorous control over strain magnitude and velocity under well standardized experimental conditions, the additional study using chemically skinned muscle fiber is clearly required.

In summary, the identification of the causes of the age-related increase in susceptibility to eccentric-induced muscle damage will be expected to have important clinical significance, because eccentric-induced muscle injury could accelerate the general symptoms of sarcopenia. However, there is no literature

investigating the muscle fiber susceptibility and their MHC isoform expression, thus it clearly needs to be investigated.

Research Questions

Numerous investigations have demonstrated that skeletal muscle damage results from eccentric contractions, and there are several studies that refer to apparent differences in susceptibility of different muscle fiber types to eccentric-induced muscle injury. Most studies reported preferential damage of type II fibers in human (Friden et al., 1983; Jones et al., 1986; Asp et al., 1998) and animal muscles (Lieber & Friden, 1988; Friden & Lieber, 1992; Macpherson et al., 1996; Vijayan et al., 2001; Brockett et al., 2002; Rader et al., 2007; Lynch et al., 2008). However, none of the human studies directly measured fiber dysfunction and most of the animal studies have been conducted on muscles rather than fibers. Even those studies that did investigate fiber mechanics assumed fibers were fast or slow instead of directly measuring their MHC isoform content or shortening velocity. Therefore, the purpose of the Chapter 2 is to investigate the relationship between fiber type and eccentric-induced muscle damage with the same rigor that has been applied to other mechanical properties of muscle cells, such as the relationship between fiber type and shortening velocity.

In Chapter 3, we tested three hypotheses, derived from the data presented in Chapter 2 and from this literature review, describing relationships between fiber

type and susceptibility to eccentric-induced muscle damage. Hypothesis 1 was based on the prevailing idea that fast muscle fibers are more suspcetable to damage than slow muscle fibers. Hypothesis 2 predicted that fibers expressing the fastest MHC isoforms, type IIx or faster, were more suseptable to damage. Finally Hypothesis 3, stated that co-expression of multiple MHC isoforms was associated with increased susceptibility to damage. By studying mouse muscle fibers, we were able to test these hypotheses across the full range of mammalian limb skeletal muscle MHC isoforms and their major hybrid combinations.

The increased susceptibility of the elderly to eccentric muscle damage is of clinical importance, because the animal studies have shown that recovery after eccentric damage is slowed (Faulkner et al., 1990; McArdle et al., 2004), or even absent (Brooks & Faulkner, 1990; Rader & Faulkner, 2006a, b), in muscles of old rodents. Therefore, in the Chapter 4, we examined the effects of high mechanical strain on the Ca^{2+}-activated force of single skinned muscle fibers prepared from vastus lateralis muscle biopsies of elderly human subjects (78 ± 2 yrs). We utilized the identical methodology with the Chapter 2. Thus we are able to highlight similarities and differences between the responses of fiber type from young and elderly subjects.

Chapter 2

Calcium-activated Force of Human Muscle Fibers Following A Standardized Eccentric Contraction

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ABSTRACT

Peak Ca^{2+}-activated specific force (force / fiber cross-sectional area) of human chemically skinned vastus lateralis muscle fiber segments was determined before and after a fixed-end contraction or an eccentric contraction of standardized magnitude (+ 0.25 optimal fiber length) and velocity (0.50 unloaded shortening velocity). Fiber myosin heavy chain (MHC) isoform content was assayed by SDS-PAGE. Post-eccentric force deficits, a marker of damage, were similar for type I and IIa fibers but 3-fold greater for hybrid IIa/IIx fibers. A fixed-end contraction had no significant effect on force. Multiple linear regression revealed that post-eccentric force was explained by a model consisting of a fiber type independent and a fiber type specific component (R^2 = 0.91). Pre-eccentric specific force was directly associated with a greater post-eccentric force deficit. When pre-eccentric force was held constant, type I and IIa fibers showed identical susceptibility to damage while IIa/IIx fibers showed a significantly greater force loss. This heightened sensitivity to damage was directly related to the amount of type IIx MHC contained in the hybrid fiber. Our model reveals a fiber-type sensitivity of the myofilament lattice or cytoskeleton to mechanical strain that can be described as follows: type IIa/IIx > type IIa = type I. If these properties extend to fibers *in vivo*, then alterations in the number of IIa/IIx fibers may modify a muscles susceptibility to eccentric damage.

INTRODUCTION

Skeletal muscles must often dissipate energy in order to slow limb movement and absorb shock. Muscles accomplish this by producing tension as they are lengthened by an external force. When preformed at high intensity or in excess, these eccentric, lengthening, or pliometric contractions can injure the muscle tissue, inducing muscle weakness, soreness, and inflammation (Proske & Allen, 2005).

Armstrong et al. (Armstrong et al., 1991) proposed that contraction-induced muscle injury was due to *an initiating event* that targeted a *population of susceptible cells*. Considerable evidence points to mechanical strain as the initiating event (Lieber & Friden, 1993; Macpherson et al., 1997; Talbot & Morgan, 1998; Patel et al., 2004). In contrast, the identification of populations of susceptible cells has lagged. One method of differentiating populations of muscle cells is based on myosin heavy chain (MHC) isoform expression. Limb muscles of small mammals express one slow (type I) and three fast (types IIa, IIx, IIb) MHC isoforms while human's express one slow (type I) and two fast (types IIa, IIx) isoforms (Smerdu et al., 1994; Ennion et al., 1995). There is a general consensus that fast muscle fibers are more susceptible to eccentric damage than slow muscle fibers (Friden et al., 1983; Jones et al., 1986; Lieber & Friden, 1988; Warren et al., 1994). However, this is not a universal conclusion (Moens et al., 1993; Consolino & Brooks, 2004) nor are the available data entirely convincing.

Histological methods used to identify injured cells may lack the sensitivity to identify fibers showing functional deficits (Lieber & Friden, 1988). To avoid this problem, functional studies have been conducted on isolated mouse soleus and extensor digitorum longus (EDL) muscles as model slow and fast muscles, respectively. However, this approach presents additional interpretational issues. First, 50% or more of the fibers comprising the "slow" mouse soleus actually express fast MHC isoforms (Stelzer & Widrick, 2003; Danieli-Betto et al., 2005). Second, mouse EDL fibers are ~60% shorter per muscle length than soleus fibers (Brooks & Faulkner, 1988). Thus, in studies that have subjected mouse soleus and EDL to similar changes in muscle length (Moens et al., 1993; Warren et al., 1994), EDL fibers may have experienced considerably greater strain than soleus fibers.

Single fiber preparations can eliminate these issues of architecture and fiber type heterogeneity. However, most single fiber studies in this area have focused on fibers obtained from predominately fast muscles (Warren et al., 1994; Balnave & Allen, 1995; Childers & McDonald, 2004; Blaauw et al., 2008; Lynch et al., 2008). A notable exception, Macpherson et al. (Macpherson et al., 1996), reported that chemically skinned fibers isolated from the fast rat EDL experienced greater reductions in peak Ca^{2+}-activated force compared to fibers isolated from the slow soleus. While the vast majority of rat soleus and EDL fibers are slow and fast types, respectively, they are not homogeneous populations nor do all fibers express a single MHC isoform (Caiozzo et al., 2003 238). This fiber type heterogeneity and

polymorphism may be a significant issue because only a sub-population of fast EDL fibers were reported to show force deficits after eccentric contractions (Warren et al., 1994). Thus, while the Macpherson et al. data provide some of the strongest evidence in support of the idea that fast fibers are preferentially damaged, the fibers were not actually typed and there may be important details of the relationship that remain to be identified.

The purpose of the present study was to examine the relationship between fiber type and eccentric-induced damage with the same rigor that has been applied to other mechanical properties of muscle cells (for instance, the relationship between fiber type and shortening velocity, see (Reiser et al., 1985a). To accomplish this, we used a maximally activated, chemically permeabilized fiber segment preparation. This preparation bypasses changes in Ca^{2+} handling or homeostasis that may occur during eccentric damage. Thus, the strength of this preparation is that damage is confined to the myofilament lattice or cytoskeleton, the sites where the injury process has been proposed to originate (Proske & Allen, 2005). Our results, obtained on the three major MHC isoform populations present in adult human skeletal muscles (type I, type IIa, and IIa/IIx “hybrid” fibers), reveal a more complex relationship between fiber type and susceptibility to eccentric damage than suggested in previous work.

METHODS

Ethical approval: The Institutional Review Board at Spaulding Rehabilitation Hospital and Harvard Medical School, Boston, MA, approved the study. All subjects were informed of the study purpose, procedures, and risks and gave their written consent to participate. The project conformed to the standards set by the Declaration of Helsinki.

Subjects. A total of 10 subjects participated in this study (5 males and 5 females; age = 25 ± 2 yr, stature = 170 ± 3 cm, body mass = 67 ± 4 kg). None of the subjects had any acute or chronic illness or injury, including any previous injuries to the joints or tissues of the lower extremities. Subjects had not participated in any regular endurance or resistance training exercise during the previous six months.

Experimental solutions. An iterative computer program was used to calculate the composition of the relaxing and activating solutions (Fabiato, 1988). Apparent stability constants were adjusted for the total ionic strength, temperature, and pH of our experiments (Fabiato, 1985). Both relaxing and activating solutions contained 7.0 mM EGTA, 14.5 mM creatine phosphate, 20.0 mM imidazole, 4 mM Mg^{2+}-ATP, and 1 mM free Mg^{2+}. The concentration of free Ca^{2+} for the relaxing and activating solution was adjusted with $CaCl_2$ to 10^{-9} M (or pCa 9.0, where pCa = -log[Ca^{2+}]) and $10^{-4.5}$ (pCa 4.5), respectively. In both solutions, the H^{+}

concentration was adjusted to pH 7.0 with KOH and the total ionic strength to 180 mmol with KCl.

Muscle biopsy and fiber skinning. Muscle specimens were obtained from the vastus lateralis by the percutaneous needle biopsy technique. The muscle specimen was immediately placed in relaxing solution (~4°C). The specimen was dissected longitudinally into several fiber bundles, containing approximately 30-50 fiber segments each. Bundles were tied with surgical silk suture to glass capillary tubes at slightly stretched lengths and chemically skinned in a solution containing 50% glycerol and 50% relaxing solution at 4°C. After 24 hr incubation, the bundles were stored at -20°C. To ensure disruption of the sarcoplasmic reticulum, skinned muscle bundles were incubated for 30 min in relaxing solution containing 0.5% of Brij-58 (polyoxyethylene 20 cetyl ether; Sigma Chemical) prior to experiments.

Experimental set-up. On the day of an experiment, a muscle bundle was transferred into a chamber containing relaxing solution and a single fiber segment was carefully isolated. The fiber segment was securely tied to titanium wires using 10-0 suture. The length of fiber suspended between the wires averaged 1.37 ± 0.02 mm for the fibers reported in this study. One wire connector extended from an isometric force transducer (Model 400, Aurora Scientific, Aurora, Ontario) and the other wire (3 mm in length) was glued to the lever arm of a high-speed motor (Model 308B, Aurora Scientific). The mounted fiber segment was suspended in one of several small glass-bottomed chambers formed in a stainless steel plate. The

fiber could be rapidly transferred from a chamber containing relaxing solution to one containing activating solution by depression and translation of the spring loaded plate. The experimental apparatus was mounted on the stage of an inverted microscope (Olympus IX70) allowing observation of the fiber segment at 450 X during data collection. Images of the fiber were obtained using a CDC camera and a scientific graphic acquisition board.

Experimental protocol. Care was taken to set initial sarcomere length (SL) as near as possible to 2.6 µm. The actual SL was confirmed by measuring 10 consecutive sarcomeres at three places along the fiber. Fiber length (FL) was measured using a stage micrometer. Fiber cross sectional area (CSA) was modeled as an ellipse and calculated from three paired width and depth measurements obtained along the length of the fiber. Fiber width was measured in the optical plane and the associated fiber depth was determined from an orthogonal image formed by a prism oriented next to the fiber. Width and depth measurements were obtained using a calibrated eyepiece micrometer.

All functional measurements were conducted at 15°C. Data were collected using a personal computer, a data acquisition board (Model AT-MIO16E-10; National Instruments, Austin, TX), and customized LabVIEW programming (Version. 7.1, National Instruments).

Baseline measurements. Baseline unloaded shortening velocity (V_o) was determined by the slack test procedure as previously described (Widrick et al.,

2002). Briefly, the fiber was transferred into activation solution (pCa 4.5), allowed to attain peak force, and rapidly slacked (within 1 ms) a per-determined distance. The time required for tension redevelopment was recorded. The procedure was repeated at different slack length steps. A straight line was fit to a plot of slack length versus time for tension redevelopment using least-squares regression. The slope of the line, relative to FL, was recorded as V_o.

Eccentric treatment. Following the slack test, fibers were assigned to treatment or control experiments. Fibers in the treatment group were subjected to a single eccentric contraction (Figure 2-1-A). This approach was pioneered by Macpherson et al. (Macpherson et al., 1996) and has since been used, either in its original form or with some modifications, by others to assess eccentric damage to skinned muscle fiber segments (Childers & McDonald, 2004; Blaauw et al., 2008; Lynch et al., 2008). The fiber was maximally activated and at peak force, lengthened by 25% of FL at a velocity of 50% V_o. The fiber was held at its final length for 100 ms and then immediately slacked to ~ 80% of its original FL in order to zero the force transducer. The fiber was relaxed and re-extended to its original FL. The fiber was then re-activated and slacked to establish post-eccentric force. Post-eccentric force activations were repeated a minimum of five times to assure a stable, reproducible response.

Pre-treatment force was defined as the peak Ca^{2+}-activated force attained prior to lengthening and post-treatment force was defined as the maximal Ca^{2+}-

activated force attained during the post-eccentric activations (Figure 2-1-B). We also measured the maximal force attained during the lengthening contraction and calculated the work done on the fiber as it was lengthened (Figure 2-1-B). All forces were measured relative to the slack baseline and then normalized to fiber CSA.

The strain magnitude studied here falls within the physiological range of *in vivo* skeletal muscle excursion (Burkholder & Lieber, 2001). While strain velocity appears to have little impact on the eccentric force deficit, at least for fast fibers (Lynch & Faulkner, 1998), standardizing strain velocity facilitated interpretation of the forces attained and work done on the fibers during lengthening.

Control experiments. Control experiments were conducted and analyzed exactly as described above except that a single fixed-end contraction substituted for the single eccentric contraction.

Fiber MHC isoform assessment. At the conclusion of an experiment, the fiber segment was removed from the wire connectors and stored at -80°C. The segment was later suspended in 20 µl of SDS sample buffer (containing 62.5 mM Tris pH 6.8, 2% SDS, 10% glycerol, 5% betamercaptoethanol, and 0.001% bromophenol blue) and denatured. The MHC composition of the fiber segment was determined using a SDS polyacrylamide gel system that consisted of a 6% separating gel and a 4% stacking gel (acrylamide:bisacrylamide = 37:1). The gels contained 30% glycerol to improve separation of MHC isoforms. A Bio-Rad mini-

Protean tetra electrophoresis system was used to run the gels at 140 V for 6 hours. A silver staining procedure was used to visualize the protein bands. Human MHC standards were prepared from pooled vastus lateralis biopsy samples and run on each gel to verify separation of all three adult MHC isoforms (Figure 2-1-C).

Quality control. A total of 165 fibers were subjected to control (fixed-end) or eccentric treatments. Two fibers were eliminated because of problems with the slack test. Of the 144 fibers subjected to an eccentric contraction, 24 fibers broke during lengthening, and 15 fibers were eliminated because they displayed partial myofibrillar tearing following the eccentric contraction. The remaining 105 fibers all completed the entire series of post-eccentric force evaluations, i.e. there was no fiber breakage during the post-eccentric activations. These post-treatment fibers often contained areas of visual sarcomere disruption. Some post-eccentric fibers did not show obvious signs of sarcomere disruption, although we did not systemically examine the entire volume of each fiber. All 19 fibers subjected to the control fixed-end contraction completed all post-treatment testing. Figure 2-2 illustrates examples of fibers identified as acceptable and unacceptable for analysis.

Statistical analysis. Statistical analysis was performed using Stata 11 (StadaCorp LP). We confirmed that there were no gender differences in baseline SL, FL, or V_o for any fiber type. Further analysis indicated that the response of fibers from male and female subjects (i.e. their pre-treatment to post-treatment

change in force) did not differ for any fiber type. We therefore collapsed data across gender for subsequent analysis.

Multiple linear regression was used to model the damage process, with post-treatment force as the independent variable of interest and pre-treatment force, the maximal force attained during lengthening, the work done on the fiber during lengthening, and fiber MHC isoform content as dependent variables. These dependent variables were chosen because they had been previously reported to be associated with post-eccentric force loss (Macpherson et al., 1996) or were central to the goals of our study.

Because of the nature of the single fiber experiments, the fibers studied from an individual subject are correlated and therefore do not represent independent observations. This violates assumptions of ANOVA and regression. Therefore, a robust variance estimator was used to adjust standard errors for this clustering of data within subjects (Hardin & Carroll, 2003). The α error rate was set at $p < 0.05$. Data are presented as mean ± SE.

RESULTS

General characteristics of the fibers studied. Baseline characteristics of the 124 single control and treatment muscle fiber segments meeting our inclusion criteria are presented in Table 2-1. There were no differences in fiber segment SL, CSA, FL, or compliance (as estimated from the displacement axis intercept of the V_o plots) across the three MHC isoform classifications. As expected, fiber V_o varied with fiber type. Specific force was 12% lower for slow vs. fast fibers.

Mechanical characteristics of the eccentric contraction. Fibers were activated, allowed to obtain their peak force, and subjected to a single standardized eccentric contraction. We quantified the maximal force attained during lengthening and the work done on the fiber. The maximal force during the eccentric contraction did not differ between fiber types although there was a tendency ($p = 0.069$) for the type I fibers to develop lower force than the other fiber types (Table 2-2). This tendency towards lower force appeared to be related to the pre-eccentric force attained by the fibers because there was no between fiber type differences when the maximum eccentric force was expressed relative to the pre-treatment force. The tendency towards a lower maximal eccentric force probably explains why the work done on the type I fibers was 6-12% less than on the fast fibers.

Ca^{2+}-activated force after an eccentric contraction. We used the change in Ca^{2+}-activated force following a single eccentric contraction as an assay of contraction-induced damage. As shown in Figure 2-3, all type IIa/IIx fibers showed

a post-eccentric force deficit. In contrast, some type I and IIa fibers showed no Ca^{2+}-activated force deficit while others clearly displayed a reduction in force.

On average, all three identified fiber types showed significant reductions in post-eccentric force (Table 2-3). Type I and type IIa fibers showed similar absolute (in kN/m^2) and relative (post to pre ratio) losses in force, although there was a trend for the IIa fibers to show a greater absolute loss in force ($p = 0.089$). In contrast, fibers co-expressing type IIa and IIx MHC showed 3-fold greater absolute and relative force deficits. Control fibers subjected to a single fixed-end contraction showed no significant change in Ca^{2+}-activated force, regardless of their MHC content (Table 2-3). Thus, the force changes following the eccentric contraction described above could be attributed to the experimental treatments per se and not to any time-dependent deterioration of the fibers.

Modeling Ca^{2+}-activated force changes after an eccentric contraction. Multiple linear regression was used to model the force changes occurring after a single standardized eccentric contraction. The best model, explaining over 90% of the variability in post-eccentric specific force was as follows ($r^2 = 0.91$, $p < 0.001$):

$$\text{post-eccentric force} = 0.84(\text{pre-eccentric force}) + b,$$

where b is +12.10 kN/m^2 for a type I fiber, +11.56 kN/m^2 for a type IIa fiber, and -3.71 kN/m^2 for a type IIa/IIx fiber. Figure 2-3 illustrates the regression lines calculated for each of these three fiber types.

Because the slope of the model was significantly less than 1.00, post-

eccentric force was dependent on the fibers pre-eccentric force but not in a direct 1:1 ratio. Within the range of pre-eccentric forces studied, those fibers with greater pre-eccentric specific force experienced greater force deficits. For every 10 kN/m^2 increase in fiber pre-eccentric specific force, the post-eccentric force deficit rose by 1.6 kN/m^2, regardless of fiber type.

When pre-eccentric specific force was held constant, important similarities and differences were evident between the three fiber types. The responses of type I and type IIa fibers to a standardized eccentric contraction were identical because their y-intercepts, or offsets, were similar (+12.10 kN/m^2 and +11.56 kN/m^2, respectively, $p = 0.660$). This effect can be readily observed in Figure 2-3 where the regression lines for these two fiber types virtually overlap.

In contrast, the response of the type IIa/IIx fibers differed, as indicated by a significantly different y-intercept (-3.71 kN/m^2, $p < 0.001$). Figure 2-3 clearly shows the greater offset for the type IIa/IIx fiber response. Type IIa/IIx fibers therefore exhibited a greater innate sensitivity to a single eccentric contraction than did type I or IIa fibers. Quantitatively, for a type I, type IIa, and type IIa/IIx fiber, all with identical pre-eccentric specific forces, the post-eccentric force deficit of the type I and IIa fibers would be similar while the deficit of the type IIa/IIx fiber would be ~15 kN/m^2 greater.

Models using the maximal force attained during lengthening or the work done during lengthening yielded poorer predictions of post-treatment force (r^2 =

0.68 and 0.64, respectively). It is likely that force and work measurements made during lengthening contain additional variability that contributes to the poor fit. Damage likely occurs as one lengthens the fibers to make these force and work measurements. The additional variability arising from the damage process is absent in measures obtained in the pre-treatment or undamaged state. High correlations between pre-treatment force with the maximal eccentric force ($r = 0.78$ to 0.85 depending on fiber type), as well as with the work done on the fiber ($r = 0.77$ to 0.87 depending on fiber type), suggest that pre-eccentric force is a surrogate marker of the stress the fiber is subjected to while lengthening (or the work done during lengthening).

Relationship between MHC isoform content and force deficits of hybrid fibers. To further evaluate how co-expression of the type IIa and IIx MHC isoforms may mediate the post-eccentric force deficit, we used densitometry to quantify the relative amounts of each isoform in all 13 hybrid fibers subjected to a lengthening contraction. A significant relationship was observed between the relative amount of total MHC present as the type IIx isoform and the loss in force after the eccentric contraction ($r^2 = 0.70$, $p < 0.001$, Figure 2-4). Thus, for the hybrid fibers, a higher expression level of the type IIx isoform was associated with a greater post-eccentric force deficit.

DISCUSSION

Using Ca^{2+}-activated skinned muscle fiber segments, we developed a model describing how each of the major fiber type populations present in human limb muscle respond to a single standardized eccentric contraction. The model reveals a fiber-type independent component and a fiber-type dependent component to the damage process. Independent of fiber type, fibers producing greater pre-eccentric specific force experienced greater post-eccentric force deficits. When this stress-related component was held constant, fibers expressing type I or IIa MHC had identical responses to the eccentric contraction, while fibers co-expressing the type IIa and IIx isoforms showed an exaggerated susceptibility. This heightened sensitivity was directly related to the amount of the IIx isoform expressed by the hybrid fiber.

The "popping sarcomere hypothesis" (Morgan, 1990; Proske & Allen, 2005) contends that sarcomere length heterogeneity during active lengthening results in a rapid over-extension of the longest and weakest sarcomeres. The spatial disturbances produced by these long sarcomeres may be propagated throughout the fiber, disrupting the cytoskeleton (Lieber et al., 1996; Koh & Escobedo, 2004), and causing secondary damage to the t-tubular and sarcoplasmic reticulum systems and to the sarcolemma (McNeil & Khakee, 1992; Takekura et al., 2001). Over-extended sarcomeres may fail to re-interdigitate (Macpherson et al., 1997), rendering them dysfunctional. Disrupted t-tubules and sarcoplasmic reticuli can impair activation

of the contractile proteins (Jones et al., 1986; Balnave & Allen, 1995; Ingalls et al., 1998). Failure of the damaged sarcolemma to exclude Ca^{2+} can result in activation of proteolytic enzymes and further losses in function (Zhang et al., 2008).

The two-component model described here was developed using a skinned fiber preparation. This preparation bypasses those mechanisms leading up to and responsible for sarcoplasmic reticulum Ca^{2+} release. Therefore, our results reveal that the susceptibility of the myofilament lattice or cytoskeleton to eccentric contraction differs between fiber types. Our model is physiologically important because, as detailed above, disturbances at the level of the myofilament lattice or cytoskeleton initiate a series of events that culminate in muscle tissue injury. Thus, the present skinned fiber approach provides novel information concerning the susceptibility of different cell types and the initiation of the damage process. However, it is unlikely that our model reveals the entire force deficit expected for each fiber type under *in vivo* conditions as alterations to mechanisms of sarcoplasmic reticulum Ca^{2+} release can contribute to the functional changes observed after eccentric contractions (Warren et al., 1993c; Balnave & Allen, 1995).

Stress-related component. Our model reveals a fiber-type independent effect of stress on the Ca^{2+}-activated force loss following an eccentric contraction. The most direct interpretation of this finding is that some component of the myofilament lattice or cytoskeleton fails at the high forces associated with an eccentric contraction. The locus of this failure is unknown but could involve

cytoskeletal, Z-disk, or intermediate filament proteins as these are known to be disrupted by lengthening contractions (see next section).

The role that stress plays in precipitating damage is controversial. The post-eccentric force deficit has been reported to be correlated with the force during the initial eccentric contraction (Warren et al., 1993a) and a stress-dependent failure model has been previously proposed to explain eccentric muscle damage (Warren et al., 1993b). However, because force and strain co-vary during lengthening contractions, others have attempted to study these properties independently. These studies have concluded that stress is not a causative factor in the injury process (Lieber & Friden, 1993; Talbot & Morgan, 1998).

Previous skinned fiber studies, also using single stretches, reported that force was a significant contributor to damage, although not necessarily the only contributor (Brooks et al., 1995; Macpherson et al., 1996). These studies varied the magnitude of stretch and the level of Ca^{2+}-activation in order to alter fiber stress. In contrast, we standardized strain and Ca^{2+}-activation and modeled the differences in Ca^{2+}-activated stress normally observed in a population of fibers. Our results indicate that this innate, but variable, property of muscle fibers influences their response to lengthening contractions.

This stress component of our model has important consequences when interpreting the response of different fiber populations to eccentric damage. The univariate data in Table 2-3 indicated a tendency for type IIa fibers to show a

greater force deficit than type I fibers ($p = 0.089$). However, using a multivariate model to hold pre-eccentric stress constant, we found that type IIa and type I fibers showed identical responses to an eccentric contraction (Figure 2-3). The trend towards a greater average post-eccentric force deficit for type IIa vs. type I fibers was simply due to the fact that on average, the type IIa fibers attained greater pre-treatment specific forces than the type I fibers. The multivariate analysis, which is based on more information than the univariate analysis, indicates that there is no innate difference between the response of type I and IIa fibers to a single eccentric contraction standardized in terms of strain magnitude and velocity.

Fiber type-related component. A novel finding of our study is the heightened sensitivity of the type IIa/IIx hybrid fibers, but not the type IIa fibers, to an eccentric contraction. Thus, the present data do not support the general consensus that fast fibers are more susceptible than slow fibers to eccentric damage. Perhaps the strongest evidence supporting this idea comes from Macpherson et al. (Macpherson et al., 1996). These authors reported that fiber segments isolated from the fast rat EDL showed greater maximal Ca^{2+}-activated force deficits than fibers isolated from the predominately slow soleus. Interpolation of the Macpherson et al. soleus data (their Figure 2-2) to the 25% strain examined in the present study yields a Ca^{2+}-activated force deficit of ~5%, in good agreement with our force deficit of 4% for type I fibers. Extrapolation of the Macpherson et al. EDL data to 25% strain yields a Ca^{2+}-activated force deficit of ~20%. However, ~55% of rat EDL fibers

have been reported to co-express the IIx and IIb isoforms and ~35% express the type IIb MHC (Caiozzo et al., 2003). It is therefore difficult to compare the Macpherson et al. fast fiber data to the type IIa and IIa/IIx fibers examined in the present study. Along similar lines, Rader et al. (Rader et al., 2007) reported greater Ca^{2+}-activated force deficits following eccentric contractions in goat levator veli palatini fibers that expressed fast compared to slow MHC. Again, it is not clear which specific fast isoforms were expressed in these fibers, complicating comparison to the present data.

There has been at least one report in the literature that sub-groups of fast fibers have different susceptibility to damage. Warren et al. (1994) was able to differentiate between two groups of fibers isolated from mouse EDL muscles that had been subjected to eccentric contractions *in vitro*: those fibers showing a severe loss in Ca^{2+}-activated force and those showing relatively normal forces. While Warren et al. did not type their fibers, we note that the proportion of fibers showing a preferential loss in Ca^{2+}-activated force (32%) is quite similar to the proportion of EDL fibers that have been reported to co-express two MHC isoforms (26% type IIx/IIb, reference (Danieli-Betto et al., 2005)). Thus, the results of Warren at al. would be consistent with our findings if their severely affected fibers were indeed hybrid fibers.

One laboratory has previously hypothesized that hybrid fibers would be more susceptible to injury than other fiber types (Vijayan et al., 1998; Vijayan et al.,

2001). While this laboratory eventually rejected their hypothesis, it is important to note that both their research question, the increased susceptibility of severely atrophied muscles to damage, and their methodology, the histological identification of damaged cells, differed markedly from the aim and approach of the present study. However, their original hypothesis concerning the susceptibility of hybrid fibers is supported by the present work.

Why are hybrid fiber more susceptible? We present two possible explanations as to why hybrid fibers show an elevated susceptibility to eccentric damage. First, expression of multiple MHC isoforms in the same fiber may promote sarcomere length heterogeneity. The kinetics of force generation differ for slow and fast fibers subjected to lengthening contractions (Stienen et al., 1992; Malamud et al., 1996). Thus, in a hybrid fiber, sarcomeres comprised of different MHC isoforms may not generate force at the same rate. At any instant during a stretch, these kinetic differences may give rise to regions of weaker and stronger sarcomeres within a hybrid fiber. These are exactly the conditions that the popping sarcomere hypothesis predicts will lead to sarcomere over-extension.

If the degree of MHC heterogeneity was responsible for the force loss, then one might expect to see a “U” shaped response in Figure 2-4, with maximum force losses in the region of maximum MHC isoform heterogeneity (a relative type IIx expression level of 0.5). However, this may be an overly simplistic interpretation. Malamud et al. (Malamud et al., 1996) found that when fibers were stretched at the

same velocity, slow fibers had greater short-range stiffness than fast fibers. Thus, slow fibers produced greater force earlier in the stretch, an effect attributed to cross-bridge kinetics. Extending the Malamud et al. work from fibers to sarcomeres, we propose that the difference in force generation between the slower and faster cross-bridges in a hybrid fiber set up a condition that would be conducive for sarcomere popping. Furthermore, a hybrid fiber expressing predominately IIa MHC has a greater number of slower and momentarily stronger cross-bridges than a hybrid fiber expressing a predominance of type IIx MHC. The first fiber, because it has fewer weak cross-bridges, may therefore experience less damage than the second fiber, which has a greater number of momentarily weaker cross-bridges. This mechanism would be consistent with the relationship illustrated in Figure 2-4.

A second hypothesis as to why hybrid fibers display a heightened susceptibility to damage focuses not on the properties of myosin per se, but on factors that co-vary with MHC isoform expression. Proteins that have been proposed to be affected by lengthening contractions, and which show fiber-type dependent variation in either their isoform expression or their intracellular concentration, include the sarcomere stabilizing protein titin, the Z-disk cross-liner α-actinin, and the Z-disk stabilizer desmin (Meyer et al.; Friden et al., 1983; Lieber et al., 1996; Chopard et al., 2001; Koh & Escobedo, 2004; Yu et al., 2004; Prado et al., 2005). Thin filament regulatory protein isoforms also co-vary with MHC isoforms (Brotto et al., 2006). Specific alterations to these regulatory protein

isoforms may be responsible for post-eccentric reductions in Ca^{2+}-sensitivity (Balnave & Allen, 1995). Our finding of a greater susceptibility of the fastest fibers to eccentric damage may reflect greater sarcomere instability due to their specific intermediate filament or cytoskeletal protein content or to preferential effects of lengthening contractions on the thin filament regulatory proteins expressed by these fibers. More work needs to be done in order to directly test the hypotheses that fiber type susceptibility to damage is due to the properties of myosin per se and/or to protein levels or isoforms that simply co-vary with myosin isoform expression.

Limitations of the current approach. Skinned fibers differ from living cells and these differences may limit the direct generalization of the present results to *in vivo* conditions. A comparison of skinned fiber studies (present study, (Macpherson et al., 1996; Lynch et al., 2008) to intact fiber experiments (Balnave & Allen, 1995) clearly show that skinned fibers are more susceptible to lengthening contractions than living cells. This is likely because skinned fibers lack the mechanical stability conferred by the endomysium.

Could this inherent fragility of skinned fibers bias our conclusions? In this study, 39 of the 144 fibers subjected to an eccentric contraction showed complete or partial breakage. We were able to rescue 34 of these fibers and identify their MHC isoform content. Based on this analysis, complete or partial eccentric breakage rates were 29% for fibers expressing type I MHC (19 out of 65 type I fibers subjected to an eccentric contraction), 15% for fibers expressing type IIa

MHC (8 out of 54 fibers), and 35% for fibers co-expressing type IIa and IIx MHC (7 out of 20 fibers). These breakage rates do not correspond to our conclusion that type I and IIa fibers show similar susceptibility to eccentric damage or with our finding that type IIa/IIx fibers display an elevated susceptibility. Thus, our conclusions are unlikely to be biased by the fiber breakage rates that occurred during the experiments.

In living fibers, the disruption of excitation-contraction coupling (Warren et al., 1993c; Balnave & Allen, 1995; Ingalls et al., 1998) and an influx of ions across the damaged sarcolemma (Zhang et al., 2008) may contribute to the observed functional defects. These factors are absent in skinned fibers and it is unknown if they would exacerbate or mask the effects reported here. Nevertheless, the use of chemically skinned fibers presents an opportunity to investigate how eccentric contractions impact the myofibrillar lattice and cytoskeleton under conditions in which other, potentially confounding, factors are eliminated or tightly controlled. This is a significant advantage of the preparation because the myofilament lattice and cytoskeleton are the sites where the damage process has been proposed to originate (Proske & Allen, 2005).

Implications of the model. Fibers expressing mixed MHC isoforms are a common phenotype in normal skeletal muscles (Caiozzo et al., 2003). In untrained humans, type IIa/IIx hybrid fibers make up 20-24% of vastus lateralis fibers (Williamson et al., 2001; Widrick et al., 2002). Type IIa/IIx fibers therefore

comprise a sizable pool of strain-sensitive muscle cells. We propose that these type IIa/IIx fibers, and possibly fibers expressing other hybrid combinations, represent the population of eccentric-contraction susceptible cells proposed to exist in normal skeletal muscles (Armstrong et al., 1991).

The plasticity of the IIa/IIx population of cells may have important implications for eccentric muscle damage. Physical activities with a substantial eccentric component decrease the proportion of hybrid fibers in the vastus lateralis (Williamson et al., 2001; Widrick et al., 2002). In contrast, activities that consist of predominately concentric contractions, even when performed at very high intensity (maximal sprint training), have been reported to have little effect on this fiber population (Parcell et al., 2005). Prior exposure to eccentric exercise, but not concentric exercise (Whitehead et al., 1998), confers a degree of protection against future eccentric damage. If our current findings regarding the susceptibility of the type IIa/IIx fibers extends to living cells under *in vivo* conditions, then activity-related changes to the size of this fiber population may be one mechanism that serves to increase or decrease a muscles susceptibility to damage by eccentric exercise.

Table 2-1. Baseline characteristics of all control and treatment fibers.

MHC (no. of fibers)	SL, µm	CSA, μm^2	FL, mm	V_o, FL/s	compliance, % FL	pre-treatment force, kN/m^2
I (54)	2.62 ± 0.01	6091 ± 278	1.43 ± 0.04	0.79 ± 0.03	2.9 ± 0.2	106 ± 2 †
IIa (54)	2.64 ± 0.01	5259 ± 292	1.30 ± 0.03	2.47 ± 0.11 *	3.4 ± 0.3	120 ± 3
IIa/IIx (16)	2.61 ± 0.01	5656 ± 539	1.38 ± 0.05	3.54 ± 0.25 *	3.1 ± 0.4	121 ± 7

Values are mean ± SE for combined control and treatment fibers. Superscripts indicate a value significantly different from all other values (* $p < 0.001$; † $p < 0.05$). Compliance was defined as the displacement axis-intercept of the slack test plot expressed as a percentage of FL. Pre-treatment force was defined as Ca^{2+}-activated force attained during either the fixed-end treatment or the fixed-end portion of the eccentric treatment as detailed in Methods. Abbreviations: MHC, myosin heavy chain; SL, sarcomere length; CSA, fiber cross-sectional area; FL, fiber length; V_o, unloaded shortening velocity.

Table 2-2. Mechanical characteristics of the eccentric treatment.

MHC	no. of fibers	pre-ecc force, kN/m^2	max ecc force, kN/m^2	max ecc force / pre-ecc force	work done, J/liter
I	46	106 ± 2 *	203 ± 5	1.89 ± 0.02	46.2 ± 1.2 †
IIa	46	121 ± 4	227 ± 7	1.90 ± 0.03	52.7 ± 1.6
IIa/IIx	13	117 ± 6	213 ± 9	1.83 ± 0.05	49.1 ± 2.1

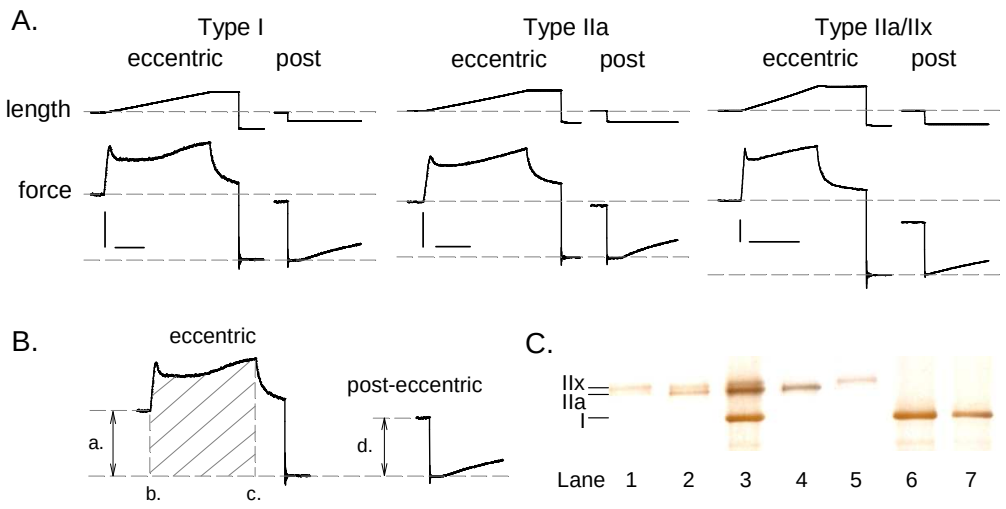
Values are mean ± SE. Abbreviations: pre ecc force, maximum Ca^{2+}-activated force prior to the eccentric treatment, max ecc force, maximum Ca^{2+}-activated force attained during the eccentric contraction. Superscripts indicate values significantly different from all other values (* $p < 0.05$; † $p < 0.05$). There was a tendency for type I fibers to produce less max ecc force than the other fiber types ($p = 0.069$).

Table 2-3. Change in peak Ca^{2+}-activated force after a single fixed-end contraction or a standardized eccentric contraction.

	change in force, kN/m^2		post force / pre force	
MHC	fixed-end	eccentric	fixed-end	eccentric
I	+ 1.5 ± 1.3 (8)	- 4.4 ± 0.8 (46) †	1.01 ± 0.01	0.96 ± 0.01 #
IIa	+ 1.8 ± 1.2 (8)	- 7.2 ± 1.1 (46) †	1.02 ± 0.01	0.94 ± 0.01 #
IIa/IIx	- 2.0 ± 1.2 (3)	- 22.0 ± 2.5 (13) † *	0.99 ± 0.01	0.82 ± 0.02 # *

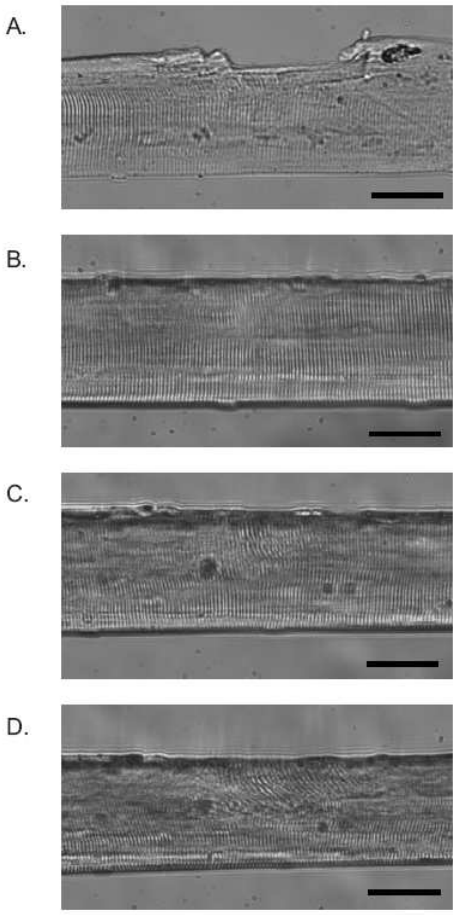
Values are mean ± SE with number of fibers in parentheses. A fixed-end contractions resulted in no significant change in absolute (kN/ms) or relative (post force / pre force) force, i.e. the mean values are not different from zero ($p = 0.117$) or 1.00 ($p = 0.114$), respectively. In contrast, an eccentric contraction resulted in a significant change in absolute and relative force, i.e. values are different from zero (†, $p < 0.001$) and from 1.00 (#, $p < 0.001$). * indicates a value significantly different from the other fiber types ($p < 0.05$).

Figure 2-1. Example of the experimental approach.



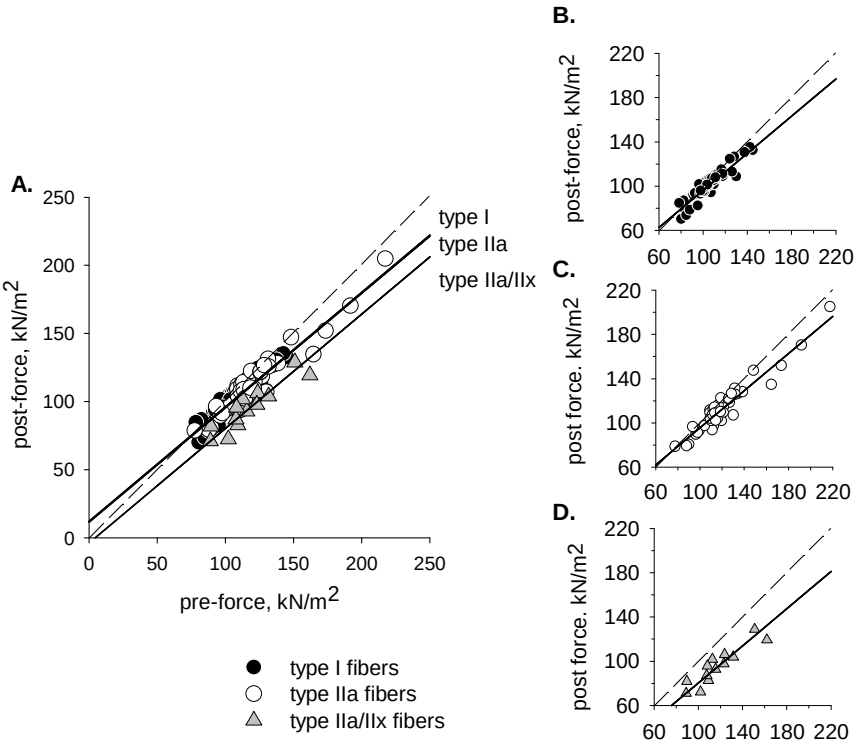
A) Position and force records obtained during eccentric and post-eccentric contractions of a representative type I, IIa, and IIa/IIx fiber. Vertical and horizontal calibration bars represent 0.20 mN and 100 ms, respectively. B) Variables obtained from the force records: pre-treatment force (a), maximum eccentric force (maximum force between time points b and c), the work done on the fiber (force-time integral between time points b and c), and post-treatment force (d). C) Example of a silver-stained polyacrylamide gel used to identify the three MHC isoforms expressed in single human muscle fiber segments. A human MHC standard was run in Lane 3. Single fiber segments were run in all other lanes.

Figure 2-2. Images obtained of fibers subjected to eccentric contractions.



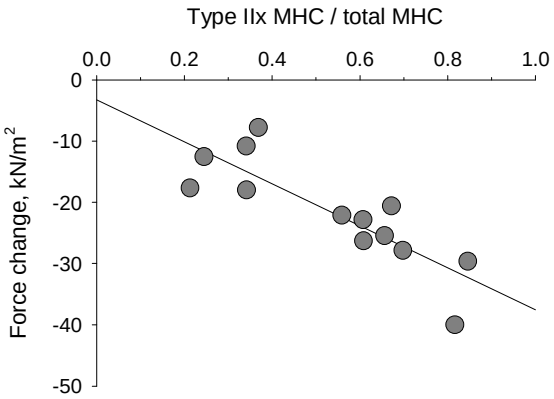
A) An example of post-eccentric myofibrillar tearing. Fibers displaying tearing were eliminated from analysis. B) A relaxed fiber prior to treatment. C) The same fiber in panel B following a single eccentric contraction (25% strain, 50% of unloaded shortening velocity). The fiber is in relaxing solution. Note focal area of disrupted sarcomeres. D) The same fiber in panel C during maximal Ca^{2+}-activation. Note widening of sarcomeres during activation. All calibration bars represent 50 µm.

Figure 2-3. Results of the multiple regression analysis.



A) Regression lines illustrating the relationship between pre- and post-eccentric force for type I, IIa, and IIa/IIx fibers. The coefficients specifying the y-intercepts are similar for the type I and IIa fibers ($p = 0.660$), but different for the type IIa/IIx fibers ($p < 0.001$). For clarity, regression lines for type I, IIa, and IIa/IIx fibers are presented individually in panels B, C, and D, respectively. In all figures, the dashed line is the line of identity. Symbols: (●) type I fibers; (O) type IIa fibers; (Δ) type IIa/IIx fibers.

Figure 2-4. Relationship between type IIx MHC isoform content and the Ca^{2+}-activated force loss of hybrid fibers.



The amount of type IIa and IIx MHC was determined from silver stained gels using densitometry. For each of the 13 hybrids fibers analyzed, the expression of the IIx isoform, relative to total MHC, was been plotted against the fiber's Ca^{2+}-activated force deficit. The force change for the IIa/IIx fibers (in kN/m^2) can be described as: -3.24 – (34.33 * (type IIx MHC / total MHC)); $r^2 = 0.70$, $p < 0.001$.

Chapter 3

Role of Myosin Heavy Chain Polymorphism in Differential Susceptibility of Muscle Fibers to Eccentric Contraction

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Abstract

We tested hypotheses regarding the influence of muscle fiber myosin heavy chain (MHC) isoform expression and susceptibility of muscle fibers to damage by high mechanical strain. Hypothesis 1 stated that fast fibers are more susceptible to eccentric contraction. Hypothesis 2 stated that the heightened susceptibility of type IIa/IIx fibers resulted from the influence of IIx or faster MHC isoform. Hypothesis 3 stated that the co-expression of a mixture MHC's increased susceptibility to damage. Chemically skinned fibers from the soleus and extensor digitorum longus (EDL) of C57BL/6 mice were studied in order to obtain the full range of mammalian limb muscle MHC monomorphic (type I, IIa, IIx, IIb) and polymorphic (I/IIa, IIa/IIx, IIx/IIb) fibers. Ca^{2+}-activated force was evaluated before and after a eccentric contraction (+.25 of fiber length, 0.5 of maximal shortening velocity) as a marker of damage. Fiber MHC isoform content was determined by SDS-PAGE. Multiple linear regression revealed that the relationship between V_o and force was not altered by an eccentric contraction, leading us to reject Hypothesis 1. To test Hypothesis 2, fibers containing "slower" MHC isoforms (type I, I/IIa, and IIa) were compared to the fibers containing "faster" MHC isoforms (type IIa/IIx, IIx, IIx/IIb and IIb). A regression model predicted similar post-eccentric force deficits between "slower" and "faster" fibers, leading us to reject Hypothesis 2. Monomorphic fibers (I, IIa, IIx, IIb MHC) and polymorphic fibers (I/IIa, IIa/IIx, IIx/IIb) were grouped to test Hypothesis 3. The model revealed that the co-expression of MHC

differentiated between fibers showing lesser and greater post-eccentric force deficits ($p < 0.001$; $r^2 = 0.97$). Therefore, we conclude that MHC polymorphism is associated with a heightened sensitivity to high mechanical strain at the level of the myofilament lattice or cytoskeleton.

Introduction

Muscle is a heterogeneous tissue, containing cells with different contractile properties, metabolic profiles, and fatigue resistance. Much of this diversity is correlated with the myosin heavy chain (MHC) isoform expressed by the cell. Mammals express nine MHC isoforms. Four of these isoforms, type I or β, type IIa, type IIx, and type IIb, are expressed in the limb muscles of small mammals (Schiaffino & Reggiani, 1996). In larger mammals, the type IIb isoform appears to be restricted to extraocular muscle fibers (Toniolo et al., 2005). Human limb muscles like the vastus lateralis therefore express only the type I, IIa, and IIx MHC isoforms (Smerdu et al., 1994; Ennion et al., 1995).

Much attention has focused on expression of MHC isoforms because of their high correlation with functional properties. For instance, fiber maximal shortening velocity is highly correlated with MHC isoform content, with type IIb > IIx > IIa > I (Reiser et al., 1985b; Larsson & Moss, 1993; Bottinelli et al., 1994b; Widrick et al., 2002). Additional functional diversity is made possible by the co-expression of multiple MHC isoforms within the same fiber (Reiser et al., 1985b; Bottinelli et al., 1994a; Widrick et al., 2002). The most common MHC combinations are type I/IIa, type IIa/IIx, and type IIx/IIb. These polymorphic or hybrid fibers can represent ≥ 25% of the total fibers in a muscle (Zhang et al. 2010; Bottinelli et al., 1994a; Williamson et al., 2001; Widrick et al., 2002; Danieli-Betto et al., 2005; Parcell et al., 2005).

It has long been recognized that some fibers are more likely to be injured or damaged by high mechanical strain than other fibers (Friden et al., 1983; Jones et al., 1986; Lieber & Friden, 1988; Warren et al., 1994). This is typically studied by subjecting muscles or fibers to eccentric or lengthening contractions in which the force producing muscle is stretched by an external force. This mode of contraction is functionally important because it enables muscles to function as brakes or shock absorbers during many common everyday activities. Eccentric contractions are clinically relevant because when performed in excess result in prolonged muscle weakness, delayed onset muscle soreness, and inflammation (Friden et al., 1983; Newham et al., 1983b; Clarkson & Sayers, 1999; Byrne et al., 2004; Proske & Allen, 2005).

Several studies have found that isolated muscles comprised predominately of fast muscle fibers show greater post-eccentric force deficits than muscles comprised of slower muscle fibers (Moens et al., 1993; Warren et al., 1994). These observations have contributed to the generally accepted hypothesis that fast muscle fibers are more susceptible to eccentric damage than slow muscle fibers. However, the isolated muscles studied are quite heterogeneous in their fiber type composition (Warren et al., 1994; Danieli-Betto et al., 2005) complicating interpretation. In addition, at least one study has failed to verify this hypothesis (Consolino & Brooks, 2004).

Issues of muscle fiber type heterogeneity can be avoided by studying skinned muscle fiber segments (Choi & Widrick, 2010; Macpherson et al., 1996; Rader et al., 2007). However, these studies have not examined a sufficiently wide range of identified fiber types to be able to reach definitive conclusions. For instance, in our own recent work on human skinned vastus lateralis fibers (Choi & Widrick, 2010), we found that when pre-eccentric force was held constant, reductions in Ca^{2+}-activated force were 3-fold greater for fibers co-expressing type IIa and type IIx MHC compared to fibers expressing either type I or type IIa MHC. These results could be interpreted as evidence that the fastest MHC isoforms, in this case type IIx, were associated with greater damage. However, an equally valid interpretation would be that the co-expression of multiple MHC isoforms within the same fiber is the critical factor.

In the present study, we subjected skinned fibers expressing the full range of mammalian limb muscle MHC isoforms (types I, IIa, IIx, IIb), and their major polymorphic combinations (type I/IIa, IIa/IIx and IIx/IIb), to a standardized eccentric contraction. We then tested three different models to determine which best described the pre- to post-eccentric change in Ca^{2+}-activated force. The models tested were based upon the following hypotheses. Hypothesis 1: fast fibers are more susceptible to damage than slow fibers. Hypothesis 2: fibers expressing the fastest MHC isoforms (type IIx or faster) are more susceptible to damage than

fibers expressing slower isoforms. Hypothesis 3: MHC polymorphism is associated with increased susceptibility to damage.

METHODS

Animals and Surgical process. Male C57BL/6 mice (Jackson Laboratory, Bar Harbor, Maine, body weight: =23 ± 1 g, N = 5) were used in this study. All mice were housed in a barrier facility under standard environmental conditions (12-12 hour light-dark cycle at 22 ºC). A standard rodent diet and tap water were freely accessible to all animals. The use of these animals was approved by the Massachusetts General Hospital Subcommittee on Research Animal Care.

Mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg/g body weight). Intact soleus and extensor digitorum longus (EDL) muscles were carefully dissected, and animals were humanely euthanized by an overdose of pentobarbital.

Fiber preparation. Immediately following dissection, the muscles were placed in 4ºC relaxing solution and dissected longitudinally into several bundles. Each bundle contained approximately 30 to 50 fibers. Dissected muscle bundles were transferred to a skinning solution, consisting of 50% relaxing solution and 50% glycerol, and stored at 4 ºC for 24 hours. The skinning solution was replaced with new skinning solution after 24 hours and the samples were stored at -20 ºC for up to 4 weeks.

Solutions. The computer program described by Fabiato (Fabiato, 1988) was used to calculate the composition of the relaxing and activating solutions. The calculations were performed using stability constants adjusted for total ionic

strength, temperature, and pH (Fabiato, 1985). Relaxing and activating solutions contained 7.0 mM EGTA, 14.5 mM creatine phosphate, 20.0 mM imidazole, 4 mM Mg^{2+}-ATP, and 1 mM free Mg^{2+}. To adjust the free Ca^{2+} concentration, $CaCl_2$ was used for both relaxing (10^{-9} M or pCa 9) and activating solutions ($10^{-4.5}$ M or pCa 4.5 where pCa = -log[Ca^{2+}]). pH was adjusted to 7.0 with KOH and total ionic strength to 180 mM with KCl.

Single fiber isolation and mounting. On the day of an experiment, a skinned muscle bundle was immersed for 30 min in relaxing solution containing 0.5% of Brij-58 (polyoxyethylene 20 cetyl ether; Sigma Chemical). This treatment ensured disruption of the sarcolemma and membrane-bound organelles. After 30 min incubation, the muscle bundle was transferred into an experimental chamber filled with the relaxing solution, and a single fiber segment was carefully isolated from the bundle. Both ends of the fiber segment were securely tied to titanium wires extending from an isometric force transducer (Model 400, Aurora Scientific, Aurora, Ontario) and a high-speed servomotor (Model 308B, Aurora Scientific). Attachment was directly to the wires using loops of 10-0 silk suture. About ~1.5 mm fiber segment was suspended in the glass-bottomed chamber. The fiber could be rapidly activated by transferring from a chamber containing relaxing solution to another chamber containing activating solution. This was accomplished by depression and translation of the chambers. The experimental apparatus was attached to the stage of an inverted microscope (Olympus IX70, Olympus America

Inc., Melville, NY) allowing the investigator to view the fiber through the glass bottom of the chambers (×600). Images of each fiber segment were taken using a digital camera and a scientific graphic acquisition board.

The temperature of the experimental solutions was controlled at 15 ºC throughout the entire experiment by a thermocouple inserted into the experimental solutions, Peltier thermoelectric devices, and by circulating water from a refrigerated bath. A personal computer and a data acquisition board (Model AT-MIO16E-10; National Instruments, Austin, TX) were used to collect functional data. Customized programs, written in LabVIEW (Ver. 6.1, National Instruments), were used for data acquisition, storage, and analysis.

Measurement of fiber dimensions. Initial sarcomere length was carefully set to ~2.6 µm by adjusting the overall segment length. The final sarcomere length was verified by measuring the length of 10 consecutive sarcomeres at three places along the fiber segment. Fiber length was then determined using a digital micrometer. The cross sectional area (CSA) of the fiber segment was modeled as an ellipse and calculated from three paired width and depth measurements obtained along the length of the fiber. The average of 3 measurements was used as the fiber CSA. Fiber width was measured in the optical plane and the associated fiber depth was determined from an orthogonal image formed by a prism oriented next to the fiber. Width and depth measurement were obtained using a calibrated eyepiece micrometer.

Measurement of unloaded shortening velocity. Unloaded shortening velocity (V_o) was determined using the slack test method (Krivickas et al., 2001; Widrick et al., 2002). The fiber was activated by transfer into activating solution (pCa 4.5), and when force reached a peak, a rapid slack step was applied. Force dropped to zero but re-developed as the fiber shortened to take up the slack. At least 5 different slack steps (all less than 20% of fiber length) were plotted versus the corresponding times required for tension re-development. To determine V_o, the plot was fit by least squares linear regression and the slope of the regression line (Δ distance / Δ time) was normalized to fiber length.

Experimental protocol. Following the slack test procedure, each individual fiber segment was subjected to a single standardized eccentric contraction. The fiber was activated and after reaching peak force, it was subjected to a 25% change in fiber length at a velocity that was 50% of V_o. The fiber was held at the final length for 100 ms followed by rapid slack step in order to obtain a force baseline. The fiber was relaxed and re-extended to its original length. Post-treatment force was measured by activating the fiber and subjecting it to a slack step when it had attained peak force. Post-treatment force was measured at least 3 times to assess reliability and consistency.

Gel electrophoresis. After the physiological assay, each single fiber segment was carefully detached from the test apparatus and transferred into 20 µl of SDS sample buffer and stored at - 20 ºC The sample buffer contained 62.5 mM

Tris (pH 6.8), 2.5% SDS, 10% glycerol, 5% betamercaptoethanol, and 0.001% bromophenol blue. Later, each fiber was denatured for 5 minutes at 100 ºC and 5 µl of denatured solute was run on a sodium dedecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) system to separate MHC isoforms. The gel system consisted of a 8% separating gel and a 4% stacking gel (acrylamide : bisacrylamide = 49 : 1). The gels included 30% glycerol to improve the separation of MHC isoforms. A Bio-Rad mini-protean tetra electrophoresis system (Bio-Rad Laboratories, Hercules, CA) was used to run the gel at 70 V for a period of ~24 hours. A silver staining procedure was used to visualize the protein bands (Giulian et al., 1983). MHC expression of each fiber segment was determined by comparison to a mouse MHC standard run on one lane of each gel. The standard was made from a mixture of mouse soleus, EDL, and diaphragm muscles. A representative silver stained 8% polyacrylamide gel, illustrating separation of all four adult limb muscle isoforms by our gel system, is shown in Figure 3-1.

Data reduction. Pre-eccentric force was defined as the force immediately prior to the imposition of the lengthening treatment. Post-treatment force was defined as the maximum force over at least 3 post-treatment activations. All force measurements were determined from the force baseline attained after a slack step. Specific force was calculated by dividing force by fiber CSA.

Hypothesis testing. Multiple linear regression was used to model the change in pre- to post-eccentric force based on the three hypotheses discussed in the

Introduction. Statistical analysis was conducted using Stata 11 (StadaCorp LP). Fibers were not considered independent observations because they were obtained from different mice. Therefore, a robust variance estimator was used to adjust standard errors for cluster of fibers within a mouse (Hardin & Carroll, 2003). The alpha rate was set at $p < 0.05$. All data are presented as mean ± SE.

Results

We studied 91 fibers from soleus and 51 fibers from EDL muscles. Figure 3-2 illustrates the distribution of fiber types from soleus and EDL muscles. A total of 7 different monomorphic or polymorphic fiber types were observed. Soleus muscles expressed all four MHC isoforms, distributed within six different fiber types (all except the monomorphic type IIb fiber type). The EDL muscle expressed IIx and IIb MHC isoforms, distributed as type IIx, IIx/IIb and IIb fibers. We also isolated one IIa and one IIa/IIx fiber from the EDL. Other groups have found similar distribution of the four MHC isoforms in C57BL/6 soleus and EDL muscles (Danieli-Betto et al., 2005).

Fiber breakage. We subjected 142 fibers to the eccentric contraction protocol. Ten of these fibers were dropped from statistical analysis because they either completely or partially broke during the eccentric treatment. However, these partially or completely broken fiber segments were recovered and run on gels. Of these 10 fibers, 3 expressed type I MHC (11% of total type I fibers), 1 expressed type IIa MHC (4%), 2 expressed type IIx MHC (10% of the total type IIx fibers), 1 expressed type IIx/IIb (17% of the total type IIa/IIx fibers), and 3 expressed type IIb MHC (11% of the total type IIb fibers). Broken down by MHC monomorphic and polymorphic fibers, the breakage rates were 8 and 4%, respectively. Because the broken fibers were not concentrated within a specific fiber type, fiber breakage rate appears to be random and would not significantly change our conclusions.

Descriptive characteristics of fibers. General characteristics of the fibers studied are presented in Table 3-1. Fiber length and sarcomere length, which are important factors for standardizing the lengthening protocol, were not different across all fiber types ($p = 0.47$). As expected, V_o varied depending upon fiber MHC isoform content. The average V_o of fibers co-expressing two MHC isoforms fell between the V_o of each of the respective individual isoforms. Fiber compliance, defined as the y-intercept of the linear relationship between slack distance and time for force redevelopment, did not differ across fiber types.

Ca^{2+}-activated force consistency. We used a time series analysis to evaluate force consistency before and after the eccentric treatment. Three consecutive pre-treatment force measurements (the last 2 slack test activations and the pre-eccentric force of the eccentric treatment contraction) and the three consecutive post-treatment measurements are shown in Figure 3-3. The variation in pre-treatment Ca^{2+}-activated force was, < 1.8%, contributing to a very slight rise in force across the three determinations, for all fiber types. Likewise the change of post-treatment force between the 7^{th} to 9^{th} contractions was < 0.8%. Therefore, Ca^{2+}-activated force was stable before and after the experimental perturbation. Thus, we have confidence that the force reduction after an eccentric contraction could be contributed to the experimental treatment per se and not to any time- or contraction-dependent effect.

Ca^{2+}-activated force before, during, and after the eccentric contraction. Fibers, expressing faster MHC isoform tended to have higher pre-eccentric specific forces than fibers expressing slower MHC isoform content (Table 3-2). Maximal force obtained during lengthening, the ratio of pre-eccentric force to maximal eccentric force, and the work done on the fiber during lengthening were all lower for fibers containing type I MHC isoform than all other fiber types ($p < 0.05$).

All fibers, regardless of fiber type showed a significant drop in Ca^{2+}-activated force following the eccentric contraction ($p < 0.001$, Figure 3-4). Average Ca^{2+}-activated force reductions ranged from 5.1 to 5.9 kN/m^2 for type I, IIa, IIx, and IIb fibers. Fibers co-expressing MHC isoforms showed ~3 fold greater reductions in force, with average values falling between 18.0 to 18.6 kN/m^2.

Mechanical properties as predictors of post-eccentric force. Previous work from our laboratory showed that pre-eccentric force was a significant predictor of post-eccentric force, i.e. greater pre-eccentric specific force was associated with greater reduction in post-eccentric force (Choi & Widrick, 2010). This relationship was found to be independent of fiber type. Therefore, we first tested whether the data from the present study followed a similar pattern. Linear regression indicated that fibers that produced greater specific force before an eccentric contraction had greater reductions in post-eccentric force independent of fiber type ($p = 0.001$, $r^2 = 0.97$; data not shown). Therefore, pre-eccentric specific force was either controlled or a component in all subsequent models.

We also tested whether the maximal eccentric force or the work absorbed by the fibers explained the pre- to post-eccentric force change. Regression models, in which pre-eccentric force was controlled, showed no relationship between maximal eccentric force and post-eccentric force or between the work done on the fiber and post-eccentric force (data not shown).

Test of Hypothesis 1. Hypothesis 1 states that fast fibers are more susceptible to eccentric contractions. Therefore, we tested Hypothesis 1 by modeling the relationship between V_o and force before and after an eccentric contraction. This approach controls for the pre-eccentric force component we have shown to be predictive of post-eccentric force. Results are plotted in Figure 3-5 and show that while the relationship between V_o and force was relatively weak ($p < 0.001$, $r^2 = 0.10$), force rose very slightly with increments in V_o ($kN/m^2 = 1.02(V_o) + 113\ kN/m^2$). Importantly, the slopes of the pre-eccentric and post-eccentric relationships were not significantly different ($p = 0.470$). Thus, an eccentric contraction reduced force uniformly across the range of shortening velocities. There was no exaggerated reduction in force at the faster velocities as predicted by Hypothesis 1. Figure 3-5 indicates little likelihood that a higher order model would adequately describe the data. We therefore found no evidence that faster muscle fibers were more susceptible to eccentric damage.

Test of Hypothesis 2. Hypothesis 2 is based on our previous work showing greater susceptibility of type IIa/IIx fibers but not type IIa fibers, to an eccentric

contraction (Choi & Widrick, 2010). To test this hypothesis, fibers expressing type IIx and/or type IIb MHC isoforms alone or in combination with other isoforms (IIa/IIx, IIx, IIx/IIb, and IIb fibers) were coded as "faster" and the remaining fibers (I, I/IIa, and IIa fibers) coded as "slower". As shown in Figure 3-6, pre-eccentric force remained a significant predictor ($p < 0.001$) in this model. However, "faster" and "slower" categories of fibers had identical responses to the eccentric contraction when pre-eccentric force was controlled (Figure 3-6-C). Thus, the model failed to differentiate between fibers showing lesser or greater changes in post-eccentric force ($p = 0.638$).

Test of Hypothesis 3. Hypothesis 3 states that a mixture of MHC within the same fiber contributes to a heightened susceptibility to damage. Therefore, fibers were coded based upon whether they expressed a single MHC isoform ("monomorphic") or a mixture of MHC isoforms ("polymorphic"). Pre-eccentric force ($p < 0.001$) and MHC polymorphism ($p < 0.001$) were significant factors predicting post-eccentric force (Figure 3-7). Thus, Hypothesis 3 was supported by our data set. This model revealed the following quantitative relationships between monomorphic and polymorphic fibers:

post-eccentric force of monomorphic fibers = (0.94 * pre-force) + 2.04

post-eccentric force of polymorphic fibers = (0.94 * pre-force) – 10.10

Greater pre-eccentric specific force was related to a greater post-eccentric force reduction regardless of fiber type (regression coefficient = 0.94). Quantitatively, for

every 10 kN/m^2 increase in pre-eccentric specific force, the force reduction following eccentric contraction would increase ~0.6 kN/m^2 regardless of fiber type. Importantly, the y-intercepts of the model were significantly different for polymorphic and monomorphic MHC groups ($p < 0.001$). For a monorphic and polymorphic fiber of identical pre-eccentric specific force, the model predicts that the polymorphic fiber would show a 12 kN/m^2 greater force reduction when subjected to a standardized eccentric contraction in comparison to the monomorphic fiber.

Discussion

The goal of this study was to identify characteristics of skeletal muscle fibers associated with a greater susceptibility to damage by high mechanical strain. We tested three hypotheses, derived from the literature (Friden et al., 1983; Jones et al., 1986; Lieber & Friden, 1988; Warren et al., 1994) and our recent work (Choi & Widrick, 2010), describing the relationship between fiber type or MHC isoform expression and susceptibility to eccentric-induced muscle damage. Hypothesis 1, that fast fibers are more susceptible to an eccentric contraction, was not support by our results. Multiple linear regression revealed that fiber V_o was not a factor contributing to a heightened susceptibility to damage (p = 0.470). Hypothesis 2, predicted that fibers expressing type IIx MHC and/or IIb MHC, alone or in combination, are more susceptible to damage. A model based on this hypothesis could not explain the force change after an eccentric contraction (p = 0.638). Thus, we rejected both Hypotheses 1 and 2. Hypotheses 1 and 2 are different ways of describing the prevailing idea that fast muscle fibers are more susceptible to eccentric-induced damage than slow fibers. Our data, based on a more rigorous examination of MHC and fiber damage than previous studies, do not support this idea.

A model based on Hypothesis 3 revealed that the co-expression of MHC differentiated between fibers showing lesser and greater post-eccentric force deficits (p < 0.001). Therefore, Hypothesis 3, which states that a mixture of MHC

isoforms confers increased susceptibility to eccentric-induced damage, is supported by our data set. We conclude that fiber MHC polymorphism, regardless of the particular isoforms expressed, is a key factor contributing to the susceptibility of skinned muscle fibers to eccentric-induced damage.

Multiple linear regression revealed that pre-eccentric specific force was always a significant factor ($p < 0.001$) in our models, thus there is a stress dependent component to the damage process that is independent of MHC isoform expression. In other words, we confirmed two characteristics of the damage process, that the magnitude of force reduction increases directly with increases in pre-eccentric specific force and that there is fiber type dependent aspect to the damage process. These two components are consistent with our previous human study (Choi & Widrick, 2010). Quantitatively, the force reduction would be increased ~0.6 kN/m^2 with 10 kN/m^2 increase of pre-eccentric specific force. Fibers containing a single MHC isoform showed average 5 kN/m^2 force reduction, otherwise fibers co-expressing MHC isoforms showed average 18 kN/m^2 force reduction following a standardized eccentric contraction.

There is some circumstantial evidence in the literature that hybrid fibers may be more susceptible to eccentric injury. Based on our previous work, the reductions in Ca^{2+}-activiated force were identical for human skinned vastus lateralis fiber, expressing type I or type IIa MHC, but 3-fold greater for fibers co-expressing type IIa and type IIx MHC (Choi & Widrick, 2010). Also, Warren et al. (Warren et

al., 1994) reported that 32% of mouse EDL skinned fibers showed preferential loses in Ca^{2+}-activated force after eccentric contractile protocol, while Danieli-Betto et al. (Danieli-Betto et al., 2005) found that 25% of the fibers in this muscle co-express two MHC isoforms (IIx and IIb). Thus, these previous findings appear to be consistent with our conclusion that hybrid fibers are the most susceptible to mechanical strain.

Our conclusion is not consistent with the general consensus that fast fibers are more susceptible to eccentric contraction than slow fibers. Some of the work supporting the general censuses used histological techniques to identify damaged fibers (Lieber & Friden, 1988; Vijayan et al., 2001). Histological approaches may lack the sensitivity to identify post-eccentric muscle dysfunction (Friden et al., 1983). For example, a force deficit of 67% was observed in muscles where only 8% of fibers exhibited visual signs of disruption (Lieber & Friden, 1988). Other studies supporting the general consensus have examined force deficits of isolated mouse extensor digitorum longus (EDL) and soleus (SOL) muscles. Functional studies utilizing the mouse SOL and EDL may be difficult to interpret because these muscles are heterogeneous in their fiber type composition (Figure 3-2 and (Zhang et al. 2010; Bottinelli et al., 1994a; Danieli-Betto et al., 2005). These muscles also have different muscle architecture. Studies that base the magnitude of an eccentric contraction on muscle length, instead of fiber length may confound valid comparisons between slow and fast muscles. For instance, fiber lengths are ~40%

shorter in mouse EDL than in mouse SOL muscles (Brooks & Faulkner, 1988). Hence, if eccentric contractions were applied based on muscle length, as several studies have done (Warren et al., 1994; Vijayan et al., 2001), then fibers in EDL muscle would be stretched 60% more than fibers in SOL muscle. The magnitude of stretch at the sarcomere level is the primary factor initiating damage (Morgan, 1990; Morgan & Proske, 2004). It is important to note that a study that failed to find the fast EDL more susceptible to damage vs. the slower mouse SOL based lengthening on fiber length, not muscle length (Consolino & Brooks, 2004). Lastly, the human studies using in vivo eccentric treatment model had indirect control over the strain magnitude at the level of the muscle fiber. These difficulties are due to differences in intramuscular fiber orientation and mechanical leverage related to joints. Finally, force deficits of whole muscles may be due to processes that are absent in our single fiber preparation.

There are only a few studies that have examined the susceptibility of slow vs. fast fibers to an eccentric contraction. One study found that fibers isolated from the EDL muscle showed greater Ca^{2+}-activated force reductions (~17% to pre-force) compared to the fibers isolated from the soleus muscle (~4% to pre-force) with 20% of strain magnitude (Macpherson et al., 1996). Even though the force reduction of slow fibers is in good agreement with the force reduction of our type I fibers, there seems to be a substantial difference between the fast fibers. However, it is important to note that ~80% of rat SOL fibers express type I MHC and only

13% of SOL fibers are hybrids. In contrast, while the EDL is made up almost exclusively of fast fibers, close to 60% of these fibers express multiple MHC isoforms (Caiozzo et al., 2003). Thus, direct comparisons of the present data, where we have identified monomorphic and polymorphic fibers, with the Macpherson et al. data is difficult. Likewise, Rader et al (Rader et al., 2007) reported greater force reduction of type II fibers vs. type I fibers from goat levator veli palatine muscle. However, this study only identified type I and II MHC isoforms, with no type II sub-groups or monomorphic and polymorphic fibers.

The single chemically skinned fiber preparation disrupts all membrane-bound structure and organelles, such as sarcolemma and sarcoplasmic reticulum, without damaging the myofilament structure. Thus, skinned fiber preparations can avoid several limitations, such as fiber type heterogeneity, while allowing the investigator control over strain magnitude and velocity under standard experimental conditions of temperature, ionic strength and activating Ca^{2+} concentration. A limitation is that the chemically skinning preparation lacks some properties of living cells, such as the excitation-contraction (EC) coupling process, may remove some proteins that confer mechanical stability to the cell, such as dystrophin, and allows soluble enzymes to diffuse from the cell, including proteolytic enzymes. Therefore, the present results may not represent the response of intact, living cell to eccentric activity. Thus, additional studies are required to investigate how living cells response to eccentric contraction under in vivo conditions.

The major advantage of the skinned fiber approach is that changes in functional properties can be unequivocally attributed to the myofilament lattice or cytoskeleton. Importantly, this is the site where the damage process is initiated (Morgan, 1990; Morgan & Proske, 2004).

What are the mechanisms responsible for heightened susceptibility of polymorphic fibers? A strong possibility is that hybrid fibers may have inherently greater sarcomere length heterogeneity than fibers expressing a single MHC isoform during an eccentric contraction. Sarcomere length heterogeneity has been proposed as the key mechanical event that initiates cell damage during lengthening contractions (Morgan, 1990; Morgan & Proske, 2004). In support of this "popping" sarcomere hypothesis, Patel et al. (Patel et al., 2004) reported that heterogeneity of sarcomeres was increased as the activated muscle bundles underwent successive stretches, and found a high correlation (r^2=.87) between sarcomere strain and the relative force deficit. Hybrid fibers contain sarcomeres expressing different MHC isoforms. Fibers expressing different MHC isoforms have different kinetic properties of force generation during lengthening (Stienen et al., 1992; Malamud et al., 1996). If these findings on fibers of different MHC content extend to sarcomeres, then multiple MHC isoforms in series within a fiber may result in increased sarcomere length heterogeneity during lengthening.

Another possible mechanism is fiber type related variation in proteins that serve to stabilize sarcomeres or maintain fiber integrity, such as titin, desmin and α-

actinin (Meyer et al.; Friden et al., 1983; Lieber et al., 1996; Chopard et al., 2001; Koh & Escobedo, 2004; Yu et al., 2004; Prado et al., 2005). Different mechanical properties of these proteins, either because of different isoform expression or different protein levels, may result in more or less compliant cytosleletal elements that contribute to sarcomere heterogeneity during an eccentric contraction.

Fibers expressing a mixture of MHC isoforms are a common phenotype in normal skeletal muscles. Several studies reported the relative proportion of hybrid fibers to range between 20 – 30% in human VL muscle (Williamson et al., 2001; Carroll et al., 2004; Parcell et al., 2005; Kohn et al., 2007) and even higher is some animal muscles (Zhang et al. 2010; Pette & Staron, 2001; Caiozzo et al., 2003; Danieli-Betto et al., 2005). Our data therefore suggests that a substantial number of muscle cells may have a heightened susceptibility to eccentric activity. This conclusion has several implications for muscle plasticity.

Hybrid fibers have been proposed to represent a transitional fiber type between populations of monomorphic fibers (Pette & Staron, 2001). What drives these fiber type conversions is poorly understood. We propose that eccentric contraction may underlie the transition of fiber types. In the model proposed by Pette and Staron (Pette & Staron, 2001), the co-expression of MHC isoforms occurs as fibers transition between fiber types. We propose a revised model by adding the y-axis ‘susceptibility to eccentric contraction’. Figure 3-6 shows the Pette and Staron model coupled with our conclusions on fiber type susceptibility to

eccentric contraction. In this proposed model, the heightened susceptibility of hybrid fibers to eccentric damage promotes their transitions to other fiber types.

Our proposed model also offers an explanation for the “repeated bout effect”. The literature consistently shows that muscles rapidly adapt to an initial eccentric exercise bout and experience less damage on a subsequent bout (Newham et al., 1987; Nosaka & Clarkson, 1995). One explanation for these observations is that weak or susceptible sarcomeres are damaged by the initial bout and adapt before the second bout (Armstrong et al., 1983; Byrnes et al., 1985; Mair et al., 1995). Our data suggests a model in which the hybrid fibers are most likely to be injured by the initial bout of eccentric activity. These damaged hybrid fibers then regenerate as MHC monomorphic fibers, which have greater resistance to damage. By the replacement of susceptible fibers following the initial injury, the muscle as a whole increases its resistance to further eccentric-induced muscle damage due to an up-regulation in the number of monomorphic fibers. As time goes on, the regulation of MHC expression in monomorphic fibers is somehow lost, and these fibers begin expressing multiple MHC isoforms. This increases their susceptibility to damage and the cycle can be repeated. This explains why resistance to eccentric exercise is lost over several months if the exercise is not repeated periodically. Our model is supported by the studies reporting a decreased portion of hybrid fibers after exercise with an eccentric contraction, such as running (Kohn et al., 2007; Harber & Trappe, 2008) or resistance training (Williamson et al., 2001; Shoepe et

al., 2003), but not after exercise involving only concentric contraction, such as 6 weeks of uphill exercise (Glaser et al. 2009) or 8 weeks of sprint cycling training (Parcell et al., 2005).

In summary, fibers containing a mixture of MHC isoforms are more susceptible to eccentric contraction than fibers expressing a single MHC isoform. We found no evidence to support the prevailing idea that faster muscle fibers are more susceptible than slower fibers. Fiber MHC polymorphism is therefore associated with a heightened susceptibility of the myofilament lattice or cytoskeleton to eccentric contraction-induced damage.

Table 3-1**.** Pre-treatment characteristics of each fiber type

MHC	No. of Fibers	FL, mm	SL, µm	V_o, FL/s	Cmpl
I	24	1.30 ± 0.03	2.61 ± 0.001	1.88 ± 0.09	2.9 ± 0.3
I/IIa	7	1.23 ± 0.09	2.60 ± 0.001	3.61 ± 0.36	4.4 ± 1.1
IIa	27	1.31 ± 0.04	2.61 ± 0.001	3.70 ± 0.22	3.1 ± 0.3
IIa/IIx	5	1.38 ± 0.09	2.60 ± 0.004	3.58 ± 0.27	3.5 ± 0.8
IIx	34	1.30 ± 0.02	2.60 ± 0.002	5.44 ± 0.21	3.2 ± 0.2
IIx/IIb	11	1.40 ± 0.06	2.60 ± 0.002	5.95 ± 0.46	2.8 ± 0.4
IIb	24	1.36 ± 0.05	2.60 ± 0.002	6.12 ± 0.17	3.2 ± 0.3

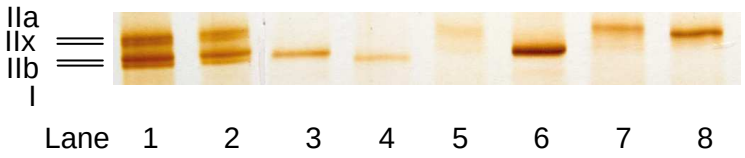
Values are mean ± SE. Abbreviations: MHC, myosin heavy chain; FL, fiber length; SL, sarcomere length; V_o, unloaded shortening velocity; Cmpl, compliance. Compliance was defined as the displacement axis-intercept of the slack test plot expressed as a percentage of FL.

Table 3-2. Ca^{2+}-activated force before, during and after the eccentric contraction

	pre-force	Eccentric contraction			pre to post force change	
	kN/m^2	max ecc force kN/m^2	max ecc force / pre-force	work done J/liter	kN/m^2	%
I	109 ± 3	219 ± 6	2.02 ± 0.04	48.5 ± 1.5	-5.1 ± 0.4	-4.8 ± 0.5
I/IIa	118 ± 7	248 ± 14	2.10 ± 0.07	54.7 ± 4.0	-18.6 ± 1.4	-15.5 ± 1.2
IIa	117 ± 3	253 ± 6	2.17 ± 0.04	55.8 ± 1.5	-5.3 ± 0.4	-4.5 ± 0.5
IIa/IIx	136 ± 11	296 ± 14	2.21 ± 0.09	62.0 ± 5.1	-18.1 ± 2.3	-13.4 ± 1.5
IIx	119 ± 2	281 ± 5	2.38 ± 0.04	61.1 ± 1.4	-5.1 ± 0.4	-4.5 ± 0.5
IIx/IIb	124 ± 2	281 ± 7	2.27 ± 0.05	61.4 ± 1.9	-18.0 ± 1.2	-14.4 ± 0.9
IIb	120 ± 2	292 ± 4	2.43 ± 0.03	62.2 ± 1.1	-5.9 ± 2.5	-4.7 ± 0.7

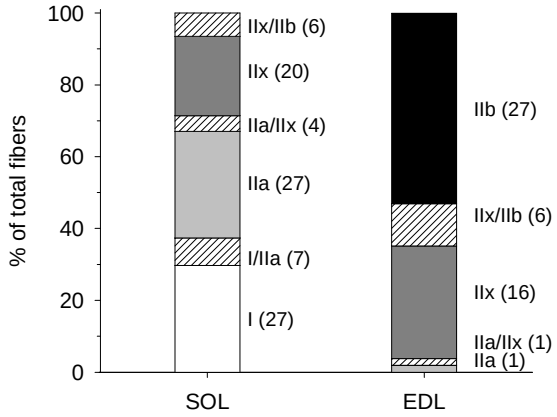
Values are mean ± SE. Pre-force was defined as Ca^{2+}-activated force attained during fixed-end portion of the eccentric treatment. Abbreviations: max ecc force, maximum Ca^{2+}-activated force attained during the eccentric contraction; max ecc force/pre-force, the ratio of maximal eccentric force to pre-eccentric force.

Figure 3-1. Example of MHC isoform identification.



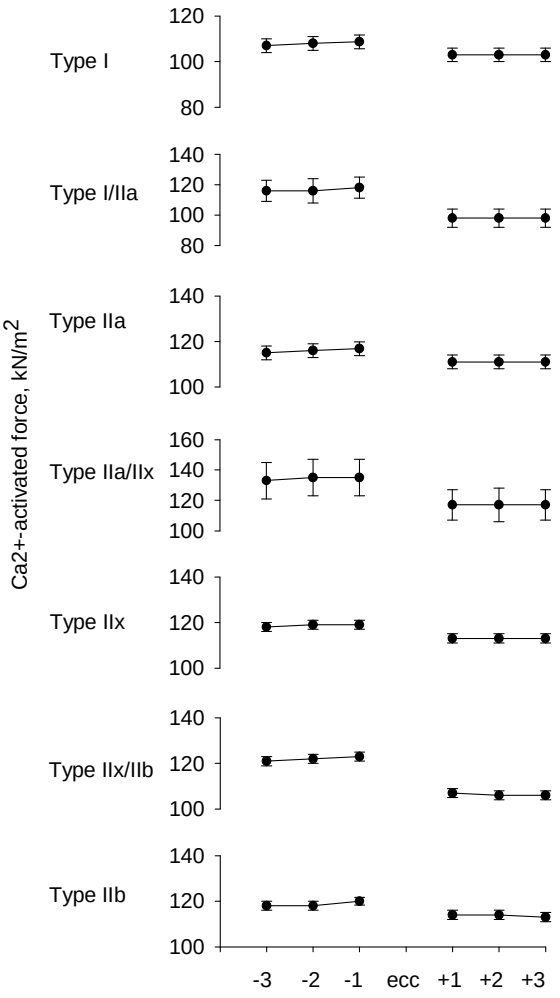
Representative silver stained polyacrylamide gel of fibers expressing type I, IIa, IIx, and IIb myosin heavy chain. A MHC standard, prepared from mouse soleus, EDL and diaphragm muscle, was run in Lanes 1 and 2. Single fiber segments were run in all other lanes.

Figure 3-2. Distribution of fibers isolated from mouse soleus and extensor digitorum longus muscles.



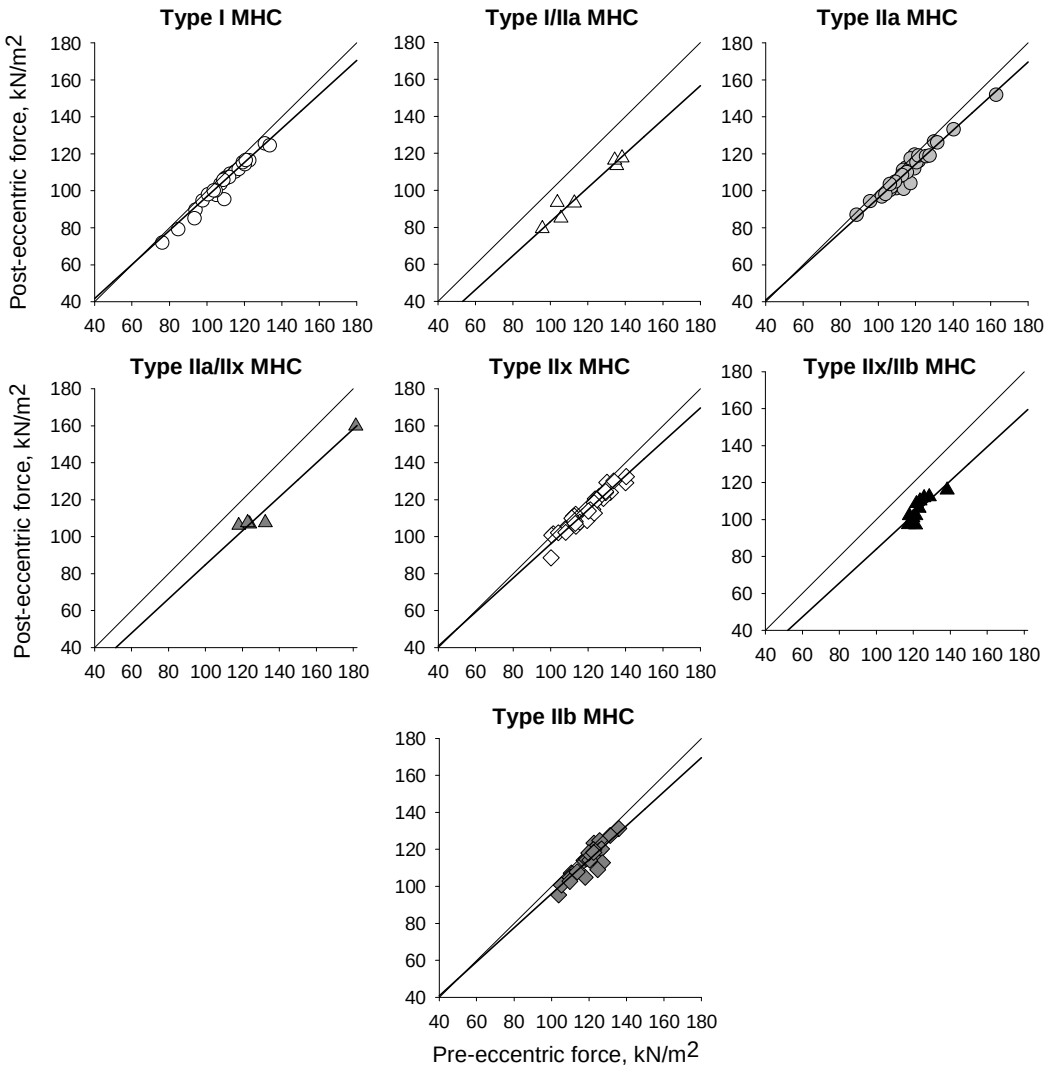
Solid areas represent the proportion of monomorphic fibers in each muscle. Hatch areas represent the proportion of polymorphic fibers in each muscle. Abbreviations: SOL, soleus; EDL, extensor digitorum longus. The numbers in parenthesis represent the number of fibers.

Figure 3-3. Ca^{2+}-activated force change during the experimental procedure.



Three consecutive pre-treatment force measurements (the 4th and 5th contractions of the slack test and the pre-eccentric force measurement) and three consecutive post-treatment force measurements. Values represent mean ± SE.

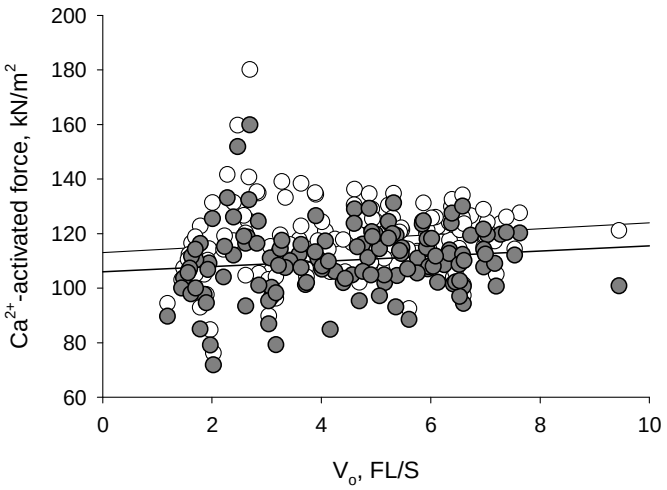
Figure 3-4. Relationship of pre- to post-eccentric force for each groups of fibers.



In all figures, the dashed line is the line of identity. For all panel, the regression line illustrate the relationship between pre- and post-eccentric force. Note the relatively lesser force reduction of the fibers, expressing a single MHC isoform content and the greater force reduction of the fibers expressing a mixture of MHC isoforms.

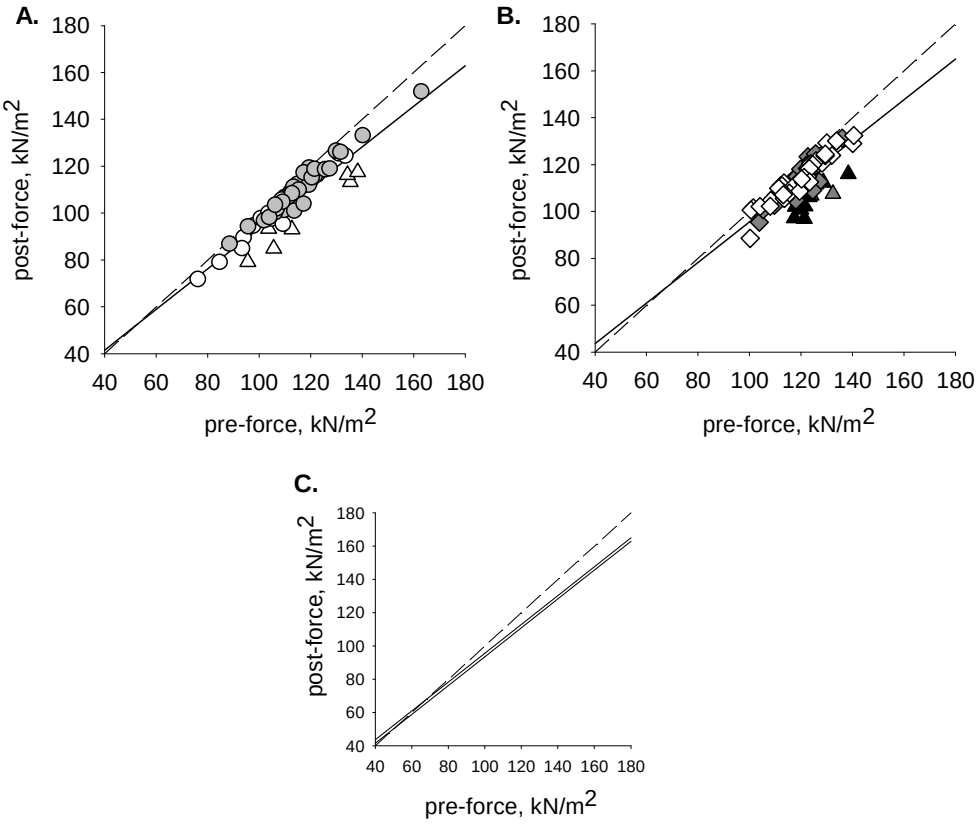
Figure 3-5. Result of Hypothesis 1

Figure 3-5. Test of Hypothesis 1.



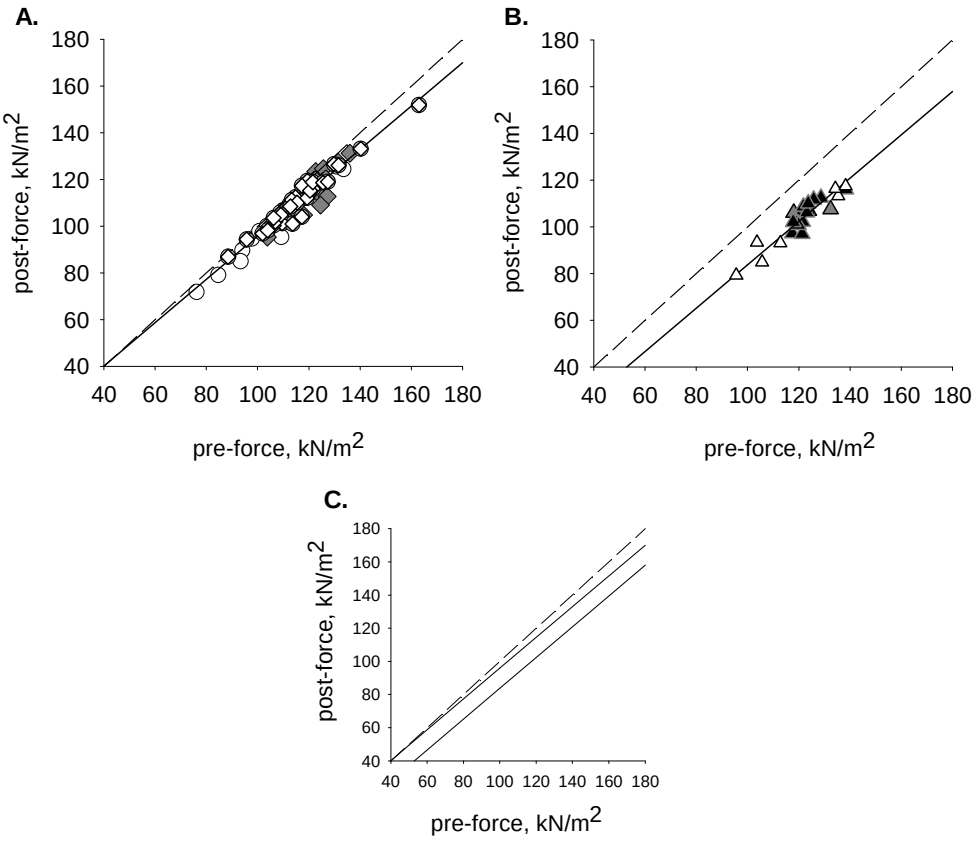
The open symbols represent pre-eccentric force and the dark symbols represent post-eccentric force. The solid line is the regression line for pre-eccentric force and the dashed line is the regression line for post-eccentric force. Abbreviations: V_o, unloaded shortening velocity; FL, fiber length

Figure 3-6. Test of Hypothesis 2



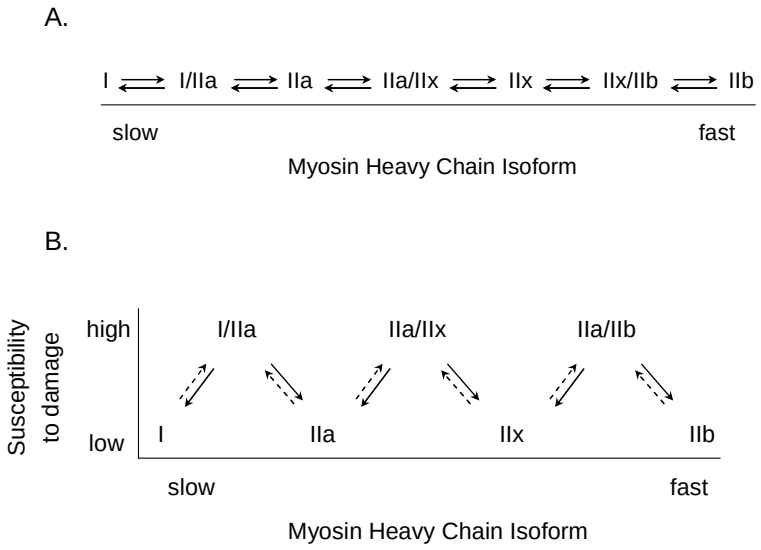
Panel A represents the regression result from the slower MHC group (type I, I/IIa, and IIa fibers). Panel B represents the regression result from the faster MHC group (type IIa/IIx, IIx, IIx/IIb, and IIb MHC fibers). For direct comparison, the regression lines for slower MHC group and the faster MHC group are combined in Panel C. Symbols represent as following: (○) type I fibers; (●) type IIa fibers; (◊) type IIx fibers; (◆) type IIb fibers; (Δ) type I/IIa fibers; (▲) type IIa/IIx fibers; (▲) type IIx/IIb fibers. In all figures, the solid line is the regression line and the dashed line is the line of identity.

Figure 3-7. Result of Hypothesis 3



In all figures, the solid line is the regression line and the dashed line is the line of identity. Panel A represent the regression result from the monomorphic MHC group, including type I, IIa, IIx, and IIb fibers. Panel B represent the regression result from the polymorphic MHC group, including type I/IIa, IIa/IIx, and IIx/IIb, and IIb MHC fibers. For clarity, regression line for monomorphic MHC group and polymorphic MHC group are combined in panels C. Each symbols are represent same as Figure 6.

Figure 3-8. Models for fiber type transitions.



Panel A shows the model proposed by Pette and Staron (Pette & Staron, 2001). Panel B is a revised Pette and Staron model coupled with our results on fiber type susceptibility to eccentric contraction. In Panel B, the solid lines and arrows represent our proposed response of fibers to eccentric exercise. The dotted lines and arrows represent an unidentified mechanism that results in the formation of polymorphic fibers.

Chapter 4

Effect of High Mechanical Strain on Force of Single Skinned Muscle Cells From Elderly Adults

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Abstract

We examined how aging affects the innate susceptibility of muscle cells to high mechanical strain. Ca^{2+}-activated force of single skinned muscle fibers prepared from vastus lateralis of elderly human subjects (N = 10, age = 78 ± 2) were measured before and after a single standardized eccentric contraction (+0.25 strain and 0.50 of maximal shortening velocity). Fiber MHC isoforms were confirmed by SDS-PAGE. Multiple linear regression revealed two components, fiber type independent component (pre-force) and fiber type dependent component (MHC expression), as important to the eccentric-induced damage process. Post-eccentric force deficits (a maker of damage) were greatest in fibers co-expressing type IIa and IIx MHC, and greater in type IIa fibers than the type I fibers. When compared to previously studied young fibers, type I fibers showed identical response to eccentric contraction, regardless of age group. Otherwise, type IIa and IIa/IIx fibers from old subject experience greater force reductions than corresponding fibers from young subjects. In conclusion, the innate susceptibility of myofilament lattice and cytoskeletal of fast fiber (type IIa and IIa/IIx fiber) of old subjects were more susceptible to a standardized eccentric contraction. Otherwise, type I fibers preserved the sensitivity to eccentric contraction regardless of aging.

INTRODUCTION

Age-related sarcopenia is characterized by a loss of skeletal muscle mass and a gradual decline in the functional properties of the tissue (Doherty, 2003; Goodpaster et al., 2006; Lynch et al., 2007; Thomas, 2007). Because of the clinical importance of these functional changes, and the demographic changes projected for the next 20 years, considerable attention has been focused on understanding how aging affects skeletal muscle contractility.

The ability to produce force under isometric or shortening conditions is reduced in old age and appears to be due to a selective atrophy or loss of fast contracting fiber types (Larsson et al., 1979; Sato et al., 1984; Lexell et al., 1988), impairments in excitation-contraction process (Delbono et al., 1995; Wang et al., 2002), and perturbations to cross-bridge function (Ansved & Larsson, 1990; Lowe et al., 2001; D'Antona et al., 2003).

In addition to isometric or shortening contractions, muscle must also actively resist stretch by an external force. This ability to resist, but not prevent lengthening, allows muscles to function as brakes or shock absorbers. These lengthening, eccentric, or pliometric contractions occur frequently during everyday activities. Force production during eccentric contractions appears to be better preserved with age than strength during isometric or shortening contractions (Vandervoort et al., 1990; Ochala et al., 2006). However, in young subjects, unaccustomed or excessive eccentric muscular activity can lead to long-lasting

weakness, soreness, and inflammation (Friden et al., 1983; Newham et al., 1983b; Clarkson & Sayers, 1999; Byrne et al., 2004; Proske & Allen, 2005)and these effects may be exacerbated with age.

Increased susceptibility followed by an impaired recovery process were reported when aged muscles were exposed to eccentric contraction, (Brooks & Faulkner, 1988; Faulkner et al., 1990; McBride et al., 1995; Brooks et al., 2001). The force deficit, a maker of damage, was two-fold greater after maximally activated single stretches of single fibers from old rodent EDL muscles compared to young (Brooks & Faulkner, 1996; Lynch et al., 2008). The contractile functions of aged-muscle did not recover for up to 2 months after eccentric contractions (Faulkner et al., 1990; McArdle et al., 2004) or it could bring about permanent loss of muscle mass and force (Brooks & Faulkner, 1990; Rader & Faulkner, 2006a, b). These characteristics of aged muscle to eccentric contraction have been exclusively observed in the studies performed on rodent muscles (Brooks & Faulkner, 1988; McBride et al., 1995; Brooks et al., 2001; McArdle et al., 2004). However, the isolated muscle or fibers prepared from these small animal model expresses high level of the IIb myosin heavy chain isoform, which is absent form of the limb muscle in larger mammals, such as humans (Smerdu et al., 1994; Ennion et al., 1995). Thus these species differences could confound generalization of animal date to humans.

The increased susceptibility of the elderly to eccentric muscle damage is of clinical importance. Animal studies have shown that recovery after eccentric damage is slowed (Faulkner et al., 1990; McArdle et al., 2004), or even absent (Brooks & Faulkner, 1990; Rader & Faulkner, 2006a, b), in muscles of old rodents. We have previously used a single skinned muscle fiber preparation to model how each of the major fiber types present in the skeletal muscles of young sedentary subjects responded to a single standardized eccentric contraction (Choi & Widrick, 2010). We found that fibers co-expressing IIa and IIx myosin heavy chain (MHC) were most susceptible to force loss, while type I and type IIa fibers showed identical force response following a single eccentric contraction. Here, using identical methodology, we have developed a similar model for fibers obtained from elderly subjects. By statistically comparing the models, we are able to highlight similarities and differences between the responses of fiber type from young and elderly subjects. Our results reveal age-dependent differences in Ca^{2+}-activated force deficits following eccentric contractions and point to difference in the biomechanics of the cytoskeleton and myofibrillar lattice as factors contributing to the heightened susceptibility of the elderly to eccentric contractions.

METHODS

Subjects. A total of 8 elderly subjects participated in this study (5 males and 3 females; mean age = 78 ± 2 yrs). Each subject completed a medical history questionnaire and was examined by a physician before entry into the study. Subjects were excluded if they had any acute or terminal illness, including coronary artery disease, neuromuscular and uncontrolled hypertension (>150/90 mmHg), had experienced a myocardial infarction or upper or lower extremity fraction in the past 6 months, or had any other unstable medical condition. None of the subjects were using drugs that affected neuromuscular function. None of the females were on estrogen replacement therapy. Subjects had not participated in any regular endurance or resistance training exercise during the previous six months. All subjects were informed of the study purpose, procedures, and risks, and each subject consented to participate in writing. We are greatful to Dr Roger Fielding at the Tufts University and Dr Walter Frontera, currently at the University of Puerto Rico, School of Medicine, for sharing muscle samples from their aging study. The Institutional Review Boards of Spaulding Rehabilitation Hospital and the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University approved the study and the procedures for recruiting and enrolling subjects.

Experimental protocol. The procedures for obtaining the muscle biopsies, preparing skinned fibers, studying fiber physiology, and detecting fiber myosin

heavy chain isoform content were identical to those used previously in our laboratory (Choi & Widrick, 2010).

Biopsy specimens were obtained from the vastus lateralis. The specimens were immediately placed in cold (4ºC) relaxing solution (for composition, see blow) where they were divided into small bundles. Bundles were tied with silk surgical suture to glass capillary tubes and chemically skinned for 24 hours in a solution containing 50% relaxing solution and 50% glycerol (4ºC). Bundles were subsequently stored at -20°C for up to 4 weeks before use. Skinned muscle bundles were incubated for 30 min in relaxing solution containing 0.5% of Brij-58 (polyoxyethylene 20 cetyl ether; Sigma Chemical) prior to experiments to ensure disruption of the sarcoplasmic reticulum.

A single fiber segment was carefully isolated from a fiber bundle and secured between an isometric force transducer (Model 400, Aurora Scientific, Aurora, Ontario) and a high-speed servomotor (Model 308B, Aurora Scientific) as previously described (Choi & Widrick, 2010). Sarcomere length averaged 2.62 ± 0.01 µm for type I fibers, 2.62 ± 0.01 µm for type IIa fibers, and 2.62 ± 0.01 µm for type IIa/IIx fibers (no differences between groups, $p > 0.05$). Fiber length (FL) averaged 1.37 ± 0.03 mm for type I, 1.38 ± 0.02 for type IIa mm, and 1.32 ± 0.03 mm for type IIa/IIx fibers (no differences between groups, $p > 0.05$). Fiber cross-sectional area (CSA) averaged 7030 ± 363 μm^2, 5704 ± 331 μm^2, and 7618 ± 911 μm^2 for type I, IIa, and IIa/IIx fibers, respectively, with the type IIa fibers

significantly smaller in CSA than the other two fiber types ($p < 0.05$). Sarcomere length, fiber length, and fiber CSA were measured as previously described (Choi & Widrick, 2010).

The experimental design and procedure were identical to previous experiments conducted in our laboratory (Choi & Widrick, 2010). The activating and relaxing solutions used for the physiological studies contained 7.0 mM EGTA, 14.5 mM creatine phosphate, 20.0 mM imidazole, 4 mM Mg^{2+}-ATP, and 1 mM free Mg^{2+},with either 0.1 µM free Ca^{2+} for the relaxing solution (pCa 9.0) or 31.6 µM free Ca^{2+} for the activating solution (pCa 4.5). Solution pH and total ionic strength were 7.0 and 180 mmol, respectively. The concentration of metals and ligands were determined using an iterative computer program (Fabiato, 1988) as previously described (Choi & Widrick, 2010). Solution temperature was continuously monitored by a small thermocouple submerged in the solution next to the fiber and all experiments were conducted at a solution temperature of 15°C. An overview of the experimental protocol is illustrated in Figure 4-1. Briefly, a slack test procedure, performed as previously described (Choi & Widrick, 2010), was used to determine pre-treatment unloaded shortening velocity (V_o). After the slack test, fibers were maximally activated, force was allowed to plateau, and the fiber was lengthened 25% of FL at a velocity of 50% of V_o. The fiber was held at the final length for 100 ms, and then slacked to ~ 80% of initial FL in order to zero the force transducer. The fiber was immediately transferred into relaxing solution and

once it had relaxed, re-extended to its original FL. Post-treatment force was measured by activating the fiber, allowing it to attain peak force, and subjecting it to a slack step ≤ 20% of FL.

Some fibers were assigned to a control treatment in order to verify that the changes observed following the eccentric treatment could be attributed to the treatment per se and not to preparation run down. Control fibers were treated exactly as described above except that a fixed-end contraction (followed by a slack step) substituted for the lengthening contraction.

Fiber segment MHC isoform content was evaluated using gel electrophoresis and silver staining as previously described (Choi & Widrick, 2010). An example of a silver stained gel, illustrating identification of all three adult MHC isoforms present in human skeletal muscle, is presented in Figure 4-2.

Definitions. The transducer zero output was used as a baseline for all force measurements. Pre-treatment force was defined as the force immediately prior to lengthening or, in the case of control fibers, the peak force attained during the fixed-end treatment contraction. Post-treatment force was defined as the maximal force attained over 4-5 post-treatment activations. All forces were normalized to the fiber's CSA.

Quality Control. Fibers were excluded from analysis if they broke or if they showed partial myofibrillar tearing at any observational time point following the experimental treatment (See Figure 4-2a of choi for an example of tearing).

Statistical Analysis. Stata 11 (StataCorp LP) was used for all statistical analyses. There were no gender differences for SL, FL, pre-treatment specific force, or V_o for any of the three fiber types. In addition, fibers from males and females showed similar force changes (in kN/m^2) following the experimental treatment. Thus, data was collapsed across gender for subsequent analysis.

Multiple linear regression was used to identify the factors contributing to the damage process. We modeled the post-treatment force (independent variable) using fiber MHC isoform content, the pre-treatment force, the maximal force attained during the eccentric contraction, and the work absorbed attained during eccentric treatment as dependent or predictor variables. These predictor variables were chosen because they have been shown in previous work to be correlated with eccentric damage (Lynch et al., 2008) and because we had used similar variables in our study of younger subjects (Choi & Widrick, 2010). A robust variance estimator was used to adjust standard errors for this clustering of data within subjects (Hardin & Carroll, 2003). The alpha rate was set at $p < 0.05$. All data are presented as mean ± SE.

RESULTS

Breakage rate of fibers. None of the 29 control fibers studied broke or tore during an experiment. In contrast, 29 fibers out of the 141 fibers subjected to the eccentric treatment showed complete or partial breakage at some point during an experiment and were eliminated from analysis. However, all of these fibers were recovered and subjected to fiber type identification. Results indicated almost identical breakage rates for type I (12 of 60 fibers, or 20%), type IIa (13 of 64 fibers, or 20%), and type IIa/IIx fibers (4 out of 17 fibers, or 24%). Thus, our results are unlikely to be confounded by any fiber type bias in fiber breakage.

Baseline characteristics of fibers. Characteristics of the control and eccentric treatment fibers are presented in Table 4-1. Ca^{2+}-activated specific force did not vary across fiber types. As expected, fibers expressing fast MHC isoforms showed faster V_o than fibers expressing the slow, type I isoform ($p < 0.05$). Fiber compliance, defined as the slack step axis intercept of the slack test plots as a percent of FL, was not different across fiber types. Specific force and V_o were similar to values for young subjects recently studied in our laboratory (Choi & Widrick, 2010). Our data are therefore consistent with the literature reporting that single muscle fiber force and shortening velocity are preserved with aging (Frontera et al., 2008; Slivka et al., 2008; Raue et al., 2009).

Mechanical characteristics of the eccentric contraction. The maximum force attained during the eccentric contraction, the work absorbed by the fiber, and

the ratio of pre-eccentric to maximal eccentric force showed no statistical difference across the three fiber types (Figure 4-3). This is probably due to our standardization of strain magnitude and velocity. Thus, variables describing the eccentric contraction per se could not be used to differentiate between fiber types.

Focal sarcomere disruption following the eccentric contraction. Light microscopy revealed regular sarcomeres spacing in relaxed and activated fibers before the eccentric treatment (Figure 4-4). Disrupted regions of sarcomeres were often visible after an eccentric contraction. These regions were comprised of weaker sarcomeres as evidenced by their distension when the fiber was Ca^{2+}-activated.

Ca^{2+}-activated force change after the eccentric contraction. The change in Ca^{2+}-activated force following a single standardized eccentric contraction was used as a marker of damage because it provides a reliable and valid measure of eccentric-induced muscle injury (Newham et al., 1983a; Faulkner et al., 1993). The force change was calculated as the difference between the pre-treatment force, attained immediately prior to the lengthening or fixed-end contraction, and the maximal post-treatment force attained during the subsequent post-treatment activations.

As shown by the gray bars in Figure 4-5, the average post-treatment Ca^{2+}-activated force of all three fiber types was significantly reduced following a single eccentric contraction ($p < 0.001$). Force was reduced 5 ± 1 kN/m^2 (4 ± 1 % of pre)

for the type I fibers, 19 ± 1 kN/m^2, (17 ± 1 % of pre) for the type IIa fibers, and 28 ± 2 kN/m^2 (24 ± 1 % of pre) for the type IIa/IIx fibers. Thus, type IIa fibers showed a 40% greater deficit than type I and type IIa/IIx fibers showd a 50% greater deficit than type I fibers. Regardless of fiber type, control fibers treated to a fixed-end contraction showed no significant decline in force (black bars in Figure 4-5). This confirms that the force change after the eccentric contraction could be attributed to the experimental treatment per se, and not to any time-dependent deterioration of the preparation.

Modeling post-eccentric Ca^{2+}-activated force. Multiple linear regression was used to model post-eccentric force from all possible combinations of pre-treatment force, fiber type, maximum eccentric force, and work absorbed during the eccentric contraction. The best model to predict post-eccentric force was obtained with pre-eccentric force and fiber type as independent variables ($r^2 = 0.91$, $p < 0.001$). This model was described by the following equation (where force is in kN/m^2);

$$\text{type I post-eccentric force} = 0.88\ (\text{pre-force}) + 8.87\ \text{kN/m}^2$$

$$\text{type IIa post-eccentric force} = 0.88\ (\text{pre-force}) - 5.26\ \text{kN/m}^2$$

$$\text{type IIa/IIx post-eccentric force} = 0.88\ (\text{pre-force}) - 14.10\ \text{kN/m}^2$$

Thus, fiber susceptibility to eccentric contraction could be described as follow: type IIa/IIx > type IIa > type I fibers.

Similarity and difference between ages on fiber susceptibility. In a previous study, using fibers from young subjects, we found that the susceptibility to eccentric contraction can be described as follows: type IIa/IIx > type IIa = type I fibers. The present study utilized the identical experimental design and approaches with previous study to make valid comparison. Thus comparison between fibers, obtained from young and old subjects was conducted by controlling an additional independent variable 'age'. The model revealed a significant interaction between fiber MHC and age on the type IIa fibers ($p < 0.001$), but type I fiber and type IIa/IIx fibers did not have the interaction effect ($p = 0.495$ and $p = 0.118$ for type I and IIa/IIx fibers, respectively). Quantitatively, the type IIa fibers from old subjects showed 10 kN/m^2 greater force deficit than corresponding fibers from young subjects when fibers were subjected to the same standardized eccentric contraction. The difference on force reduce magnitude between age groups were +0.3 kN/m^2 and -4.2 kN/m^2 for the type I and type IIa/IIx fibers. The developed equations can be expressed by following;

	Young	Old
type I post-eccentric force =	0.88 (pre-force) + 7.67	+ 0.3 kN/m^2
type IIa post-eccentric force =	0.88 (pre-force) + 6.87	- 10.06 kN/m^2
type IIa/IIx post-eccentric force =	0.88 (pre-force) – 5.33	- 4.23 kN/m^2

DISSCUSSION

The present study examined the effects of high mechanical strain on the Ca^{2+}-activated force of single skinned muscle fibers prepared from vastus lateralis muscle biopsies of elderly human subjects (78 ± 2 yrs). The use of chemically skinned permeabilized single fibers allowed us to apply an eccentric contraction, standardized in terms of stain magnitude and lengthening velocity, to maximally activated cell segments and to subsequently assess the MHC isoform content of these segments. This approach, which bypasses strain-sensitive mechanisms of cell activation (Balnave & Allen, 1995), enabled us to evaluate how the myofilament lattice responded to high mechanical strain. Because contraction-induced injury to the muscle tissue has been proposed to originate at the level of the sarcomere (Proske & Allen, 2005), this approach reveals information about how the injury process is initiated in different populations of muscle cells.

A multiple regression analysis revealed two components, pre-eccentric specific force and fiber MHC expression, as important to the eccentric-induced damage process. The first of these components was independent of fiber type. Thus, higher pre-treatment specific force was associated with a greater post-eccentric treatment force deficit (regression coefficient = 0.88, $p < 0.001$). This result is consistent with our previous work examining the susceptibility of single muscle fibers obtained from young subjects (25 ± 2 yrs) to the same standardized eccentric contraction (Choi & Widrick, 2010). For every 10 kN/m^2 increase in a fibers

specific force, the force deficit following a standardized eccentric contraction increases by 1.2 kN/m^2 for old fibers and 1.6 kN/m^2 for young fibers, regardless of fiber MHC isoform expression. Thus, the magnitude of force deficit increases directly with increases in pre-eccentric specific force, and this characteristic seems to be conserved with aging, and perhaps even reduced (regression coefficient = 0.84 for young vs. 0.88 for old).

In addition to a non-specific fiber specific force component, the model predicts a significant fiber type dependence to the damage process. Graphically, this is indicated by the different y-intercept terms, or offset, in Figure 4-6. The offsets of the model (y-intercepts) were significantly different for each fiber type ($p < 0.05$). Thus, in addition to the fiber-type independent component described above, there was a fiber type dependent component to the injury process with the three groups of fibers all showing a different susceptibility to damage. Quantitatively, type I fibers showed the least force deficit, type IIa fibers an intermediate deficit that was ~ 14 kN/m^2 greater than type I, and type IIa/IIx fibers with the greatest deficit. Thus, when pre-eccentric force was held constants the relationship between the force deficit and fiber MHC isoform could be described as (from most susceptible to least susceptible) type IIa/IIx > type IIa > type I. This contrasts to our earlier work on young subjects in which the relationship was type IIa/IIx > type IIa = type I.

To further investigate these age-related differences, we statistically compared our models developed for young and old subjects. Using this age-combined approach, we are able to quantify differences between the two subject populations for each fiber type (Figure 4-7). This analysis revealed significant interactions between age and fiber MHC isoform content. Thus, when pre-eccentric specific force was held constant, post-eccentric force deficits for type I fibers are not different for 25 and 78 years olds ($p = 0.993$). Nor was there an age-related difference in the response of the type IIa/IIx fibers. In contrast, there is a significant age-related interaction in the response of type IIa fibers to a single eccentric contraction ($p < 0.001$). Quantitatively, type IIa fibers from old subjects showed a 10 kN/m^2 greater post-eccentric force deficit compared to type IIa fibers from young subjects (when pre-eccentric specific force was held constant).

The primary conclusion drawn for our age-combined modeling is that the myofibrillar lattice and/or cytoskeleton of those fibers expressing the type IIa MHC isoform becomes more vulnerable to high mechanical strain in old age. On the other hand, the resistance of the myofibrillar lattice and/or cytoskeleton of type I fibers to contraction-induced injury is preserved well into the 8^{th} decade of life, as is that of the most sensitive type IIa/IIx fibers.

To our knowledge, the present study is the first to examine how aging affects the innate susceptibility of the three main fiber type populations expressed in adult lower limb skeletal muscles to an eccentric contraction. Previous work has

shown that predominately fast lower limb muscles of old laboratory rodents experience greater post-eccentric force deficits than muscles of either young or adult animals (Zerba et al., 1990; Brooks & Faulkner, 1996; Lynch et al., 2008). Consistent with the present work, at least a portion of this deficit has been attributed to events occurring at the level of the force-producing or force-transmitting components of the cell (Brooks & Faulkner, 1996; Lynch et al., 2008). For instance, Lynch et al (Lynch et al., 2008) found a 5% greater Ca^{2+}-activated force deficit for aged muscle fibers, prepared from 26 month old rat EDL muscle, after eccentric contraction compared to 6 month young rat. Therefore, our finding of an average 8% greater force reduction for the fibers from elderly subject than fibers from young subjects, are consistant with their findings. The small difference on force reduction rate between the present and Lynch study may contribute to the discrepancy in species and/or fiber type difference, because the majority of fibers making up the rodent EDL express the IIb myosin heavy chain isoform (Stelzer & Widrick, 2003; Danieli-Betto et al., 2005), an isoform which is absent of humans (Smerdu et al., 1994; Ennion et al., 1995). Therefore, not only these functional approaches have limited by the fiber type heterogeneity, but also these species differences could confound generalization of animal data to humans.

While animal studies have consistently reported greater evidenced of eccentric-induced damage to ankle flexors of old vs. young animals, human studies have not reached the same consensus. Studies performed on the knee extensors

have revealed greater eccentric-induced damage in old adults (Manfredi et al., 1991). In contrast, the elbow flexors appear to retain their resistance to eccentric contraction into old age (Dedrick & Clarkson, 1990) or even show greater resistance than young (Lavender & Nosaka, 2006a; Lavender & Nosaka, 2006b). Our data provide a potential explanation for these findings. The vastus lateralis shows an increase in hybrid fibers during aging (Klitgaard et al., 1990; Andersen et al., 1999). If our results extend to intact fibers, then an increase in this population would increase the susceptibility of the muscle to eccentric contraction in old age. In contrast, the biceps branchii shifts toward a slower phenotype with aging, resulting in greater expression of slow fibers (Monemi et al., 1999). These fibers are not only the most resistant to damage, but they show no deterioration in resistance with age. Thus, previous discrepancies in the literature regarding the susceptibility of muscle from elderly subject to eccentric exercise may be the result of differences in fiber type distributions across lower and upper limb muscles coupled with the age-related differences and similarities in fiber type susceptibility to damage reported here.

It is well known that age-related skeletal muscle atrophy is characterized by a preferential atrophy of fast fibers (Sato et al., 1984; Lexell et al., 1988). Also there is an increase the co-expression of myosin heavy chain isoforms in old human vastus lateralis muscle (Andersen et al., 1999). These characteristics are illustrated in Figure 4-8. Taken together to our finding, type IIa and IIa/IIx fibers of old adults

may be injured by common activities that require muscle to perform eccentric contraction, such as walking, down stairs, lowering heavy objects, or recovering from a slip of fall, because the population of susceptible cell (gray area in Figure 4-8) substantially increase from ~20 % to ~55 % with aging. Thus, the novel finding of present study, the heightened susceptibility of type IIa fibers and hybrid (IIa/IIx) fibers to eccentric contraction may have etiological relationship to this selective loss of fast fibers with aging. Furthermore, it becomes more critical when it coupled with the impaired recovery process following eccentric contraction when the old adults has injured by eccentric contraction. For instance, the contractile function of aged-muscle did not recover for up to 2 months after eccentric contractions (Faulkner et al., 1990; McArdle et al., 2004) or it could bring about permanent loss of muscle mass and force (Brooks & Faulkner, 1990; Rader & Faulkner, 2006a, b). This lack of recovery is thought to be attributed to impaired muscle regeneration (Brooks & Faulkner, 1990; Rader & Faulkner, 2006a, b), resulting from the reduced number of satellite cells, shortened telomeres, replicate senescence (Gibson & Schultz, 1983; Renault et al., 2002).

Chemically skinned muscle cell is that fibers were studied in a non-physiological setting, for example excitation and contraction (EC) coupling process had been eliminated, and the skinning process may remove or allow efflux of structural or enzymatic protein that may be involved in the damage process. In addition, fibers are maximally activated where in vivo, different fiber types may be

differentially recruited by the nervous system, altering their apparent susceptibility to injury. Otherwise, the human in vivo setting involved all membrane structure and EC coupling process, but they have motor unit recruitment issue and indirect control of strain magnitude.

Using chemically skinned fibers we have identified age-related similarities and in the innate sensitivity of the myofilament lattice or cytoskeleton to the high mechanical strain produced by lengthening contractions. Multiple linear regression reveals that these force producing and force transmitting structure respond to fiber stress independently of fiber type and age, i.e. the greater the pre-stress, the greater the loss in force. When pre-stress is controlled the resistance to strain is preserved in type I and IIa/IIx fibers well into senescence. While fibers expressing type IIa MHC exhibit a heightened susceptibility to strain in old age. These finding could explain why some muscle of old adults are more sensitivity to damage by eccentric exercise.

Table 4-1. Baseline morphological and functional characteristics of all fibers used in analysis

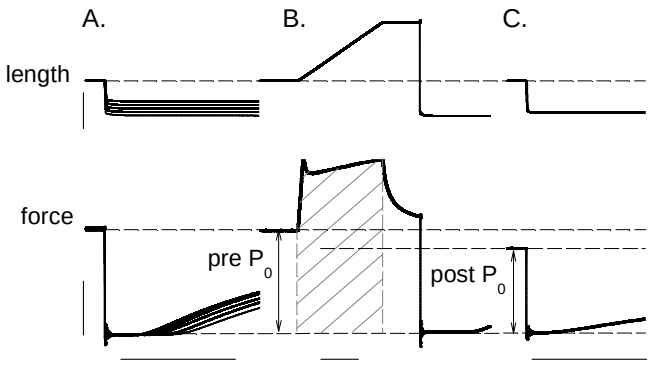
Fiber MHC (no. of fibers)	V_o (FL/s)	Compliance (%FL)	Force (kN/m^2)
I (59)	$0.75 \pm 0.03^*$	3.5 ± 0.2	115 ± 3
IIa (65)	2.62 ± 0.14	3.3 ± 0.2	117 ± 2
IIa/IIx (17)	2.97 ± 0.30	3.8 ± 0.4	119 ± 7

Values are mean ± SE.

Fibers obtained from vastus lateralis biopsies from 5 male and 3 female subjects.

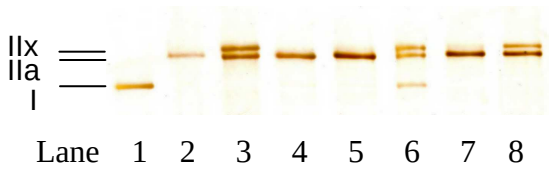
* indicates a value significantly different from the other fiber types ($p < 0.05$). Abbreviations: MHC, myosin heavy chain; V_o, Unloaded shortening velocity; FL, fiber length.

Figure 4-1. Experimental protocol.



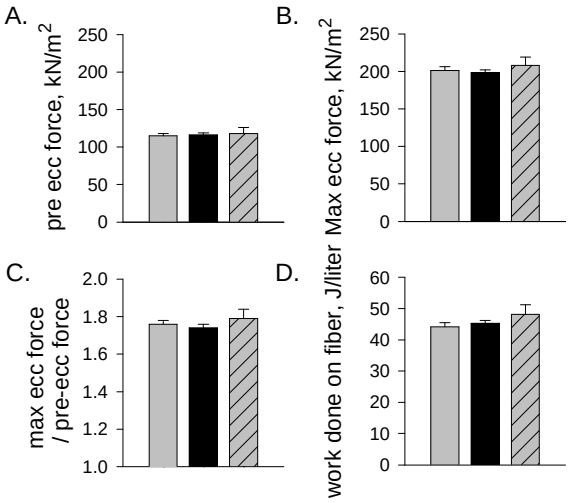
Panel A shows 5 superimposed length steps and the corresponding superimposed force responses. The time for force redevelopment was plotted against the slack length step and fit by linear regression to yield V_o (per fiber length). In panel B, the same fiber was activated and lengthened 25% of fiber length at a velocity of 50% V_o, held at the final length for 100ms, and then slacked to zero the transducer. Panel C shows one post-treatment force evaluation for the same fiber. As in Panel A, a slack step used to establish a consistent force baseline. Pre-treatment and post-treatment force (P_0) were evaluated as shown. The shaded area under the lengthening portion of the force record in Panel B represents the work done on the fiber. Control fibers were treated in an identical manner except that the fiber was not lengthened during treatment in Panel B. Vertical and horizontal calibration bars represent 200 µm for length, 0.5 mN for force, and 100 ms for time, respectively.

Figure 4-2. Identification of MHC isoform content in single fibers



Representative silver stained 6% polyacrylamide gel illustrating identification of type I, IIa, and IIx myosin heavy chain in skinned segments of human vastus lateralis fibers. Each lane, except lane 6, contains a single muscle fiber segment. Lane 1: type I; lane 2: type IIa; lane 3: type IIa/IIx; lane 4 and 5: type IIa; lane 6: human MHC standard; lane 7: type IIa; lane 8: type IIa/IIx.

Figure 4-3. Mechanical characteristics of the eccentric treatements.



A peak Ca^{2+}-activated force prior to eccentric treatment. B. Maximal Ca^{2+}-activated force attained during the eccentric contraction. C. ratio of maximal eccentric force and maximal pre-eccentric force. D. Work done on the fiber during the eccentric contraction. Values are mean ± SE. Gray, black, and gray with line is for type I, IIa, and IIa/IIx fiber, respectively.

Figure 4-4. Representative fibers images before and after an eccentric contraction under relaxed and Ca^{2+}-activated conditions.

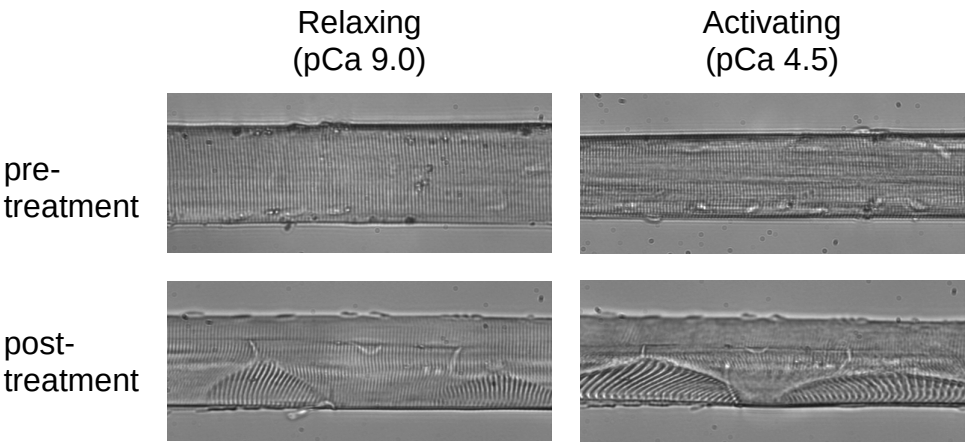
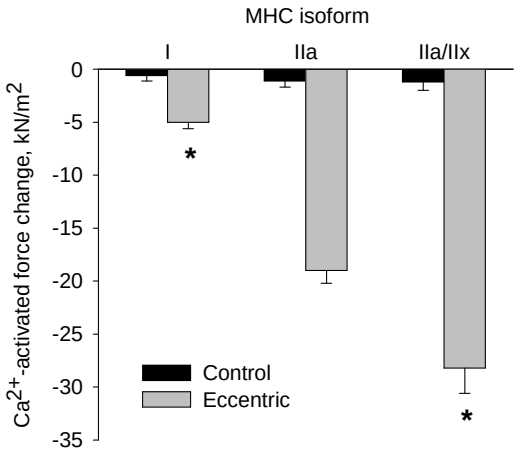
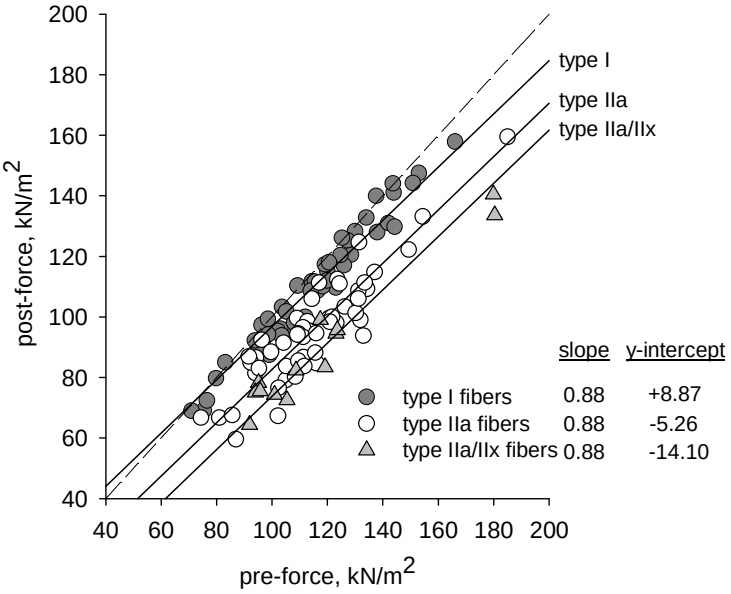


Figure 4-5. Ca^{2+}-activated force change for each fiber type following the experimental treatment.



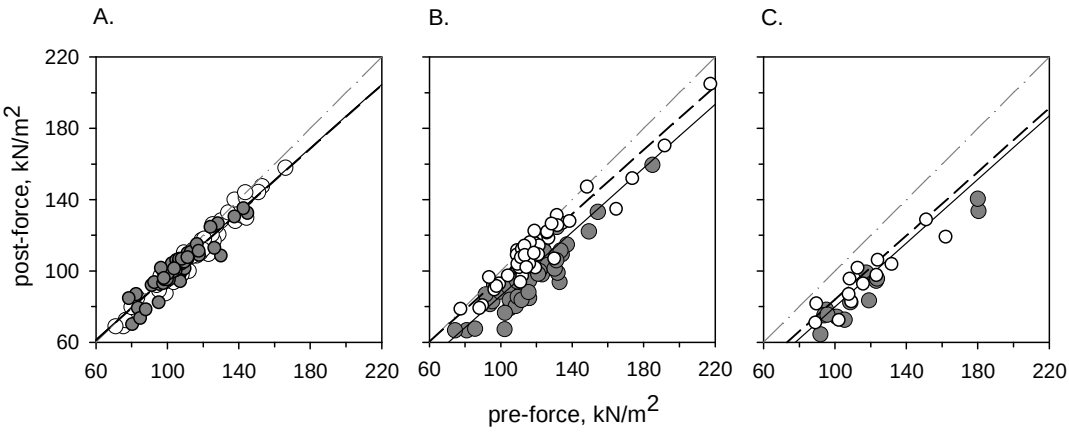
* represents significant difference from type IIa fibers ($p < 0.001$ for type I fibers, and $p < 0.05$ for type IIa/IIx fibers). Abbreviations: MHC, myosin heavy chain.

Figure 4-6. Multiple linear regression results.



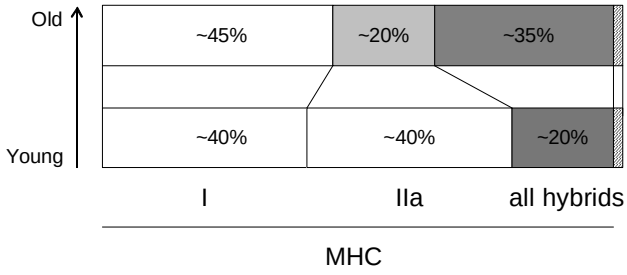
Over 91% of variability in post-eccentric force could be explained by a regression model that used pre-eccentric force and fiber MHC isoform content as independent variables. Shown are regression lines for each fiber type when pre-eccentric force is held constant. The dashed line is the line of identity. Y-intercept for the type IIa fibers was significantly different from that of the type I ($p < 0.001$) and the type IIa/IIx fibers ($p < 0.05$)

Figure 4-7. Comparison of results from young and old subject populations.



Gray points and dashed regression line represent results of young subjects reported by Choi and Widrick. Black point and solid regression line represent data from present study. Panel A, type I fibers. Panel B, type IIa fibers, Panel C, type IIa/IIx fibers. Multiple linear regression revealed a significant interaction between fiber type and age ($p < 0.001$). With pre-eccentric force controlled no difference was detected between the response of young type I and old type I or between young IIa/IIx and old IIa/IIx. However type IIa fibers, from old subjects showed a significant different response to eccentric contraction compared to the response of young subjects.

Figure 4-8. Schematization model.



Model illustrating the fiber type shifting and populations of susceptible cells with aging. Dark gray area represents the population of most susceptible cells, light gray area represents the population of intermediate susceptible cells, white area represents the population of least susceptible cells. The hatched area represents the proportion of fibers containing IIx MHC, but it was not used in the percent calculations due to their very low expression level. The fiber population of young based on Widrick, et al., 2002 and Williamson, et al., 2001. Old age fiber population based on Klitgaard, et al., 1990 and Larsson, et al., 1997.

Chapter 5.

Conclusions

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Conclusions

Musculoskeletal disorders and injuries are the most common health problem in the US, affecting one in every two adults and accounting for almost 8% of the US gross domestic product in treatment costs and lost wages (American Academy of Orthopaedic Surgeons, 2008). Activity-induced muscle injury and dysfunction have been identified as key components of musculoskeletal injuries. These injuries often occur following eccentric contractions, when the muscle is under tension and stretched by a force that is greater than the force generated by the muscle. Many daily activities require muscles to perform eccentric contractions, including walking (or running) downhill or down stairs, lowering heavy objects, and landing from a jump. Injuries often occur when these activities are performed at high intensity or for prolonged periods of time.

The etiology of contraction-induced injuries is thought to consist of two components: an *initiating event* that targets a *population of susceptible cells*. Much evidence has accumulated pointing to mechanical strain as the initiating event. In contrast to these advances in defining the initiating event, the identification of susceptible cells has lagged, resulting in a gap in our understanding of the mechanisms of injury. Limited data suggest that fibers isolated from predominately fast muscles experience greater force deficits after eccentric contractions than fibers isolated from slower muscles. However, the relationship between fiber MHC isoform content and eccentric-induced dysfunction has never been examined with

the same rigor that has been applied to other mechanical properties of muscle cells. Thus, the present research investigated force change following a single eccentric contraction and evaluated how these changes were related to the fibers myosin heavy chain (MHC) isoform content.

In the Chapter 2, Ca^{2+}-activated skinned muscle fiber segments, prepared from healthy untrained young subjects (25 ± 2 yrs, N = 10) were subjected to an eccentric contraction standarized in terms of strain magnitude (0.25 of fiber length) and lengthening velocity (0.50 of maximum shortening velocity). Maximal force was measured before and after a single eccentric or fixed-end contraction and fiber MHC isoform content was identified by gel electrophoresis. A model was developed using multiple linear regression to describe how each of the major fiber type populations present in human limb muscle responded to a single eccentric contraction. The best model revealed a fiber type independent factor (pre-treatment force) and a fiber type dependent factor (MHC expressions) to the damage process. Regardless of fiber type, fibers generating greater pre-eccentric specific force showed greater post-eccentric force deficits. When this pre-treatment force was controlled, fibers expressing type I or IIa MHC had identical force change after the eccentric contraction, whereas fibers co-expressing the type IIa and IIx MHC isoforms showed a 3-fold greater force deficit. This increased susceptibility was directly related to the amount of IIx MHC isoform expressing by the hybrid fiber. Thus, our results suggest that the co-expression of two different MHC isoforms in

the same fiber, or the presence of the fastest MHC isoform, predisposed the fiber to injury.

The results from Chapter 2 raised two testable hypotheses regarding the mechanism responsible for heightened susceptibility of type IIa/IIx hybrid fiber. First, type IIa/IIx fibers may be more susceptible due to the influence of the fast IIx MHC isoform. We formulated this as a more general form of Hypothesis 1, that faster fibers are more suscpetable to damage, and a more specific Hypothesis 2, that fibers containing type IIx MHC or faster were more susceptible to damage. The alternative hypothesis is that type IIa/IIx hybrid fibers are more susceptible because they contain a mixture of myosins. This was expressed as Hypothesis 3, MHC polymorphic fibers are more suscpetable to damage than MHC monomorphic fibers. In Chapter 3 we tested these three hypotheses utilizing soleus and extensor digitorum longus muscles from C57BL/6 mice. The use of mouse muscles allowed us to examine the full range of mammalian limb muscle MHC, including all of the monomorphic MHC fibers (type I, IIa, IIx, IIb) and the most common polymorphic MHC fibers (I/IIa, IIa/IIx, IIx/IIb). Ca^{2+}-activated force of single skinned muscle fibers was evaluated before and after a standardized eccentric contraction (0.25 of fiber length 0.50 of maximal shortening velocity). Damage was quantified as the pre- to post-eccentric change in Ca^{2+}-activated force. Multiple linear regression revealed that shortening velocity or expression of the fastest MHC isoforms were not factors contributing to heightened susceptibility to damage. Thus we rejected

Hypotheses 1 and 2. The test of Hypothesis 3 revealed that the co-expression of MHC differentiated between fibers showing lesser and greater post-eccentric force deficits ($p < 0.001$; $r^2 = 0.97$). Therefore, Hypothesis 3 that a mixture of MHC isoforms confers increased susceptibility to eccentric-induced damage, was supported by the data set. We concluded that MHC polymorphism is associated with a heightened sensitivity to high mechanical strain at the level of the myofilament lattice or cytoskeleton. These findings are novel. The well-known plasticity of this hybrid population may underlie adaptation of muscle to eccentric contractions. In addition, the heightened susceptibility of hybrid fibers to eccentric damage drives or promotes their transitions to other fiber types.

In Chapter 4, we examined the effects of high mechanical strain on the Ca^{2+}-activated force of single skinned muscle fibers prepared from vastus lateralis muscle biopsies of elderly human subjects (78 ± 2 yrs). Experimental approaches were identical to those used in Chapter 3 so that we could make valid comparisons to the young data of Chapter 2. This approach revealed information about how the damage process is initiated in different populations of muscle cells and at different ages. The result revealed that type I fibers from elderly adults showed identical Ca^{2+}-activated force reductions as the type I fibers from young adults. Likewise, force deficits for the type IIa/IIx fbers were not different for young and old. However, type IIa fibers from old subjects showed ~ 4-fold greater Ca^{2+}-activated force reduction after a single standardized eccentric contraction compared to the

corresponding fibers from young subjects. Therefore, these novel finding provide a possible mechanistic explanation for why some muscle of old adults are more sensitivity to damage by eccentric exercise.

The use of chemically permeabilized fibers allowed us to apply an eccentric contraction, standardized in terms of strain magnitude and velocity, to individual cell segments of know fiber MHC isoform content. The major advantage of the skinned fiber approach is that changes in functional properties can be unequivocally attributed to the myofilament lattice or cytoskeleton. Importantly, this is the site where the damage process is initiated (Morgan, 1990; Morgan & Proske, 2004). However, a limitation of the skinned fiber approach is that fibers are studied in an artificial milieu (although one thought to represent the intracellular milieu). In addition, the skinning process may remove or allow efflux of structural (e.g. dystrophin) or enzymatic (e.g. calpain) proteins that may be involved in the damage process. Finally, fibers are maximally activated where in vivo, different fiber types may be differentially recruited by the nervous system, altering their apparent susceptibility to injury. Therefore, additional studies need to address the response of living cells to in vivo eccentric exercise.

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