Role of the calcium-calpain pathway in cytoskeletal damage after eccentric contractions

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1Muscle Physiology Laboratory, Department of Rehabilitation Sciences, Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong; 2Muscle Cell Function Laboratory, School of Medical Sciences and Bosch Institute, University of Sydney, New South Wales, Australia; and 3Musculoskeletal Research Laboratory, Department of Orthopaedics and Traumatology, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong

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Zhang BT, Yeung SS, Allen DG, Qin L, Yeung EW. Role of the calcium-calpain pathway in cytoskeletal damage after eccentric contractions. J Appl Physiol 105: 352–357, 2008. First published May 22, 2008; doi:10.1152/japplphysiol.90320.2008.—The mechanism(s) underlying eccentric damage to skeletal muscle cytoskeleton remain unclear. We examined the role of Ca2+ influx and subsequent calpain activation in eccentric damage to cytoskeletal proteins. Eccentric muscle damage was induced by stretching isolated mouse muscles by 20% of the optimal length in a series of 10 tetani. Muscle force and muscle damage was induced by stretching isolated mouse muscles by 20% of the optimal length in a series of 10 tetani. Muscle force and membrane disruption, was evident 20 min after stretch. These markers were apparent after 5 min that accelerated over the next 60 min. Increased titin immunostaining, thought to indicate damage to titin, was evident 10 min after stretch, and fibronectin entry, indicating membrane disruption, was evident 20 min after stretch. These markers of damage also increased in a time-dependent manner. Muscle force was reduced immediately after stretch and continued to fall, reaching 56 ± 2% after 60 min. Reducing extracellular calcium to zero or applying leupeptin minimized the changes in immunostaining of cytoskeletal proteins, reduced membrane disruption, and improved the tetanic force. These results suggest that the cytoskeletal damage and membrane disruption were mediated primarily by increased Ca2+ influx into muscle cells and subsequent activation of calpain.

cytoskeleton; muscle damage; calcium-activated protease; calpain

For example, in extensor digitorum longus (EDL) muscle, immunolabeling of desmin is reduced within 5 min of eccentric exercise, and this loss is correlated with a decline in contractile force (21). Increased titin immunostaining, thought to indicate damage (see discussion), has also been observed ~30 min after EC, and at a similar time fibronectin, which is normally extracellular, appears inside the muscle cells, indicating defects in membrane integrity (14, 21). In rat muscle, the cytoskeletal protein dystrophin is disrupted even after a single EC experimental protocol (22). In dystrophin-deficient (mdx) mice, stretch-induced muscle fiber disruption and force decline are much more severe (10, 11, 37). Although cytoskeletal proteins have been implicated in stretch-induced muscle injury (3, 7, 14, 21), the underlying mechanisms remain unclear.

It has been proposed that calcium plays a role in EC-induced muscle damage (19, 21, 34, 37). Resting levels of intracellular calcium ([Ca2+]i) increase significantly after EC protocols in single muscle fibers (2) and whole muscle (23). Furthermore, [Ca2+]i is elevated in resting muscle fibers from mdx mice, and even more so after mechanical stretch (16, 37). Removing extracellular calcium prevents the rise in resting [Ca2+]i and ameliorates the decline in muscle force, suggesting that Ca2+ influx contributes to eccentric damage (37). Calcium increases protein turnover in muscle through activation of proteases, phospholipases, and lysosomal enzymes (8). However, it is more likely that calpain, a calcium-activated neutral protease, mediates the muscle damage that follows EC (6). Activities of μ- and m-calpain are increased in rat skeletal muscle following prolonged treadmill running (5). Autolytic activation of calpain-3 is increased in human muscle biopsies subjected to eccentric exercise (25). Application of a calpain inhibitor, leupeptin, largely prevents the excitation-contraction uncoupling and the titin disruption that follows elevated [Ca2+]i in toad muscle (9262).

MUSCLE DAMAGE following eccentric contractions (EC) is characterized by an immediate, prolonged loss of muscle strength and plays an important role in exercise training (9) and sports injuries (29). Many factors contribute to the loss of muscle force, including abnormalities in excitation-contraction coupling (2, 18, 36), damage to force-generating structures such as actin and myosin (31), and damage to the force-transmitting system that is composed of cytoskeletal proteins (3, 7, 21, 22).

Muscle cytoskeletal proteins maintain the structural integrity of the myofiber, transmit force generated by actomyosin interactions, and are ubiquitous throughout the muscle cell. These proteins are susceptible to damage during eccentric exercise.

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muscle following EC. These abnormalities were alleviated by removing extracellular calcium or by sustained application of leupeptin to isolated muscle fibers. This study supports the hypothesis that damage to muscle cytoskeleton following EC is primarily attributed to calcium-activated calpain proteolysis.

MATERIALS AND METHODS

Animals. Male BALB/c mice (age 10–14 wk, body wt 30–40 g) were used. Animal care procedures and the experimental protocol were approved by the Animal Ethics Committee of the Hong Kong Polytechnic University.

Muscle dissection and mounting. Mice were killed by cervical dislocation. The skin overlying the lateral aspect of the hindlimb was then dissected from the thigh to the ankle. The proximal and distal tendons of the EDL muscle were carefully isolated and gripped with T-shaped aluminum foil microclips. The muscle was then transferred to an experimental chamber (capacity 1.5 ml). One end of the tendon was attached to a hook connected to the lever arm of a position feedback motor (300B-LC, Aurora Scientific), and the other end was attached to a force transducer (BG-10g, Kulite Semiconductor Products). The force transducer was clamped to a mechanical micro-manipulator that allowed the muscle length to be adjusted.

Solutions. During the experiments, EDL muscle was superfused with Krebs solution composed of (in mM) 121 NaCl, 5 KCl, 1.8 CaCl₂, 0.5 MgCl₂, 0.4 NaH₂PO₄, 24 NaHCO₃, 5.5 glucose, and 0.1 EDTA. This solution was bubbled with 95% O₂-5% CO₂, maintaining zero Ca²⁺-lacking solution, or leupeptin-containing solution was analyzed by repeated-measures ANOVA. The percentage of cells showing abnormal protein immunostaining in Ca²⁺-lacking, zero Ca²⁺-, or leupeptin-containing solution was analyzed by repeated-measures ANOVA. The percentage of cells showing abnormal protein immunostaining in Ca²⁺-containing, zero Ca²⁺-, or leupeptin-containing solution was analyzed by one-way ANOVA with Bonferroni correction for multiple comparisons. P < 0.05 is considered significant. Values are expressed as means ± SE.

RESULTS

Effect of stretched contractions on tetanic force in isolated EDL mouse muscle. The changes in force following isometric and eccentric contractions were determined (Fig. 1). Tetanic force was transiently depressed immediately after 10 isometric contractions, due to muscle fatigue, but recovered to the control level within 5 min. Following 10 eccentric contractions, the force at 1 min declined to 76 ± 4% (n = 5, P < 0.01), and continued to decline, reaching 56 ± 2% at 60 min (n = 5, P < 0.001).

Fig. 1. Effect of removing extracellular Ca²⁺ or applying leupeptin, a calpain blocker, on mouse extensor digitorum longus (EDL) muscle tension following eccentric contractions (EC). Muscle force was normalized to the prestretch level (baseline, ■). After stretched contractions (●), muscle force was significantly lower than after isometric contractions (Iso, ▲). Application of leupeptin (◆) or solution lacking Ca²⁺ (zero Ca²⁺, ○) partially recovered relative muscle force after stretched contractions. *P < 0.05, significant difference between untreated and treated (Ca²⁺-lacking solution, or leupeptin treated) groups after stretch. Values are expressed as means ± SE.
Fig. 2. Effects of leupeptin and lack of extracellular calcium on eccentric contraction-induced changes in the staining pattern of mouse EDL muscle cytoskeletal proteins. A: cross sections of EDL muscle immunostained for different cytoskeletal proteins 30 min post-isometric contractions or eccentric contractions (EC) in standard Krebs solution, solution lacking Ca\(^{2+}\) (zero Ca\(^{2+}\)) or leupeptin-containing solution. *Same fiber in serial sections. Arrows indicate dystrophin rupture. Scale bar, 50 \(\mu\)m. B: stretch-induced changes in muscle cytoskeletal proteins expressed as the percentage of muscle fibers affected (affected fibers/total no. of fibers per section). *\(P < 0.05\), significant difference between untreated and treated (solution lacking Ca\(^{2+}\), or leupeptin treated) groups after stretch. Values are expressed as means \(\pm\) SE.
Effects of a calpain inhibitor and zero Ca$^{2+}$ on tetanic force.

To test the hypothesis that cellular damage induced by EC is mediated by Ca$^{2+}$ influx and subsequent calpain activation, EC were performed in the presence of 100 μM leupeptin, a calpain inhibitor, or zero-Ca$^{2+}$ solution applied after the last EC. Application of leupeptin 1 h before and after stretch significantly attenuated the decline in muscle force. Force at 30 min and at 60 min (69 ± 4%) poststretch were larger than in the absence of leupeptin ($n = 5$, $P < 0.05$). Similarly, tetanic force was also greater in the absence of extracellular Ca$^{2+}$ at 15, 30, and 60 min (76 ± 3%) ($n = 5$, $P < 0.05$) (Fig. 1).

Effects of a calpain inhibitor and zero Ca$^{2+}$ on the levels of cytoskeletal proteins. Representative examples of the effects of leupeptin and zero Ca$^{2+}$ on EC-induced changes in the staining of cytoskeletal proteins dystrophin, desmin, and titin, and the extracellular matrix protein fibronectin are shown in Fig. 2A. Figure 2B shows the time course of average changes after stretched contractions: either the loss of dystrophin and desmin staining or the increase of fibronectin and titin staining. Desmin staining is completely absent in some cells and patchily absent in others and quite normal in others. Dystrophin staining is located in the surface membrane and becomes disrupted in an irregular fashion. Titin and fibronectin staining is not detectable within the muscle cell under control conditions and becomes detectable throughout the muscle cell or in a patchy way after stretch. Note that increased loss of desmin staining and dystrophin disruption were evident at 5 min, whereas the increase in staining for titin and fibronectin was not observed until 10 min and 20 min, respectively. For example, Fig. 3 shows the dystrophin, desmin, and fibronectin immunostaining at 5 min post–eccentric contractions and illustrates abnormal dystrophin staining, absence of desmin staining, but no increase in fibronectin staining. Reducing extracellular Ca$^{2+}$ to zero prevented the loss of desmin staining (2.1 ± 0.7%, $P < 0.05$) as did application of leupeptin (1.7 ± 0.6%, $P < 0.01$). EC-induced changes in dystrophin, titin, and fibronectin staining were not significantly affected by zero extracellular Ca$^{2+}$ or leupeptin until 10–20 min.

At 30 and 60 min after stretch, all the changes in protein immunostaining were highly significant, and application of zero extracellular Ca$^{2+}$ solution or leupeptin prevented all the immunostaining abnormalities (desmin, $P < 0.001$; dystrophin, $P < 0.001$; titin, $P < 0.01$; and fibronectin, $P < 0.01$). These data confirm previous studies that EC can lead to significant loss or damage to cytoskeletal proteins. Desmin and dystrophin were damaged earlier and more severely than titin. Not all desmin- or dystrophin-abnormal fibers displayed fibronectin staining, perhaps representing differences in the cytoskeletal damage pathway compared with the membrane damage pathway. The calpain inhibitor leupeptin and zero-extracellular Ca$^{2+}$ solution prevented stretch-induced abnormalities of cytoskeletal proteins, including desmin, dystrophin, and titin, as well as extracellular matrix fibronectin.

**DISCUSSION**

The aim of this study was to determine whether the calcium-activated protease calpain contributes to damage of cytoskeletal components in mouse skeletal muscle following EC. Following repeated EC, loss of immunolabeling for desmin and dystrophin and increase in titin and fibronectin staining were largely prevented and the decline in force was partly prevented by applying the calpain inhibitor leupeptin or removing extracellular calcium.

Muscle cytoskeleton in stretched muscles. Muscle cytoskeleton contains a transverse (consisting primarily of desmin) and longitudinal (consisting of titin and nebulin) filament system (28, 32), by which the generated force is transmitted from the sarcomere to the fiber surface. In addition there is the dystrophin-glycoprotein complex, which completes these connections through the sarcolemma to the extracellular matrix. Changing this complex by lengthening active muscles may also affect the function of other membrane-associated proteins, including ion channels, which may in turn lead to elevated [Ca$^{2+}]_i$. Consistent with previous studies (7, 21, 22), immunolabeling for desmin and dystrophin declined rapidly, whereas the increase in immunological staining of titin and in intracellular fibronectin staining was somewhat slower. Temporal differences in the damage to these cytoskeletal proteins probably relate to their sensitivity to calcium-protease activity, although differences in spatial distribution or functional role during eccentric contractions may also contribute. The increase in titin staining after stretch was first described by Lieber et al. (21) and was thought to result from greater exposure of antigenic sites after partial hydrolysis. Titin is very readily proteolyzed by calpains (17) in response to increased Ca$^{2+}$ concentration or stretch (35). The protease calpain-3 cleaves titin at the PEVK region near the COOH terminus (20), which might involve the binding site of titin antibody used in this study. Of course, a possibility that progressive disruption of extramyofibrillar network would make antibody accessible to intramyofibrillar components more easily is not excluded. Absence of fibronectin staining in fibers that had reduced desmin staining suggests that many fibers with damaged cytoskeletal proteins retained their membrane integrity (Fig. 3). We also confirmed earlier studies showing that eccentric damage leads to a reduction in muscle force (2, 15, 21, 37).

Intracellular calcium and cytoskeletal damage in stretched muscles. Although [Ca$^{2+}]_i$ was not measured in the present study, other reports show that [Ca$^{2+}]_i$ was significantly ele-

![Fig. 3. Changes in the staining pattern of mouse EDL muscle cytoskeletal proteins dystrophin and desmin and extracellular matrix fibronectin 5 min post-EC. Absence of desmin and disruption of dystrophin staining was evident at 5 min post-EC, whereas fibronectin staining was not detectable intracellularly within the same fiber. *Same fiber in serial sections. Arrows indicate dystrophin rupture. Scale bar, 50 μm.](image-url)
vated after 10 min and remains elevated 1.2- to 2.5-fold for hours (2, 18) and even days (13, 23) following EC protocols. In mdx fibers, EC increases the resting [Ca\textsuperscript{2+}], decreases tetanic [Ca\textsuperscript{2+}], and decreases muscle force, and these changes could be alleviated by excluding Ca\textsuperscript{2+} from the medium (37). Excitation-contraction can become uncoupled, and titin degradation occurs following elevation of [Ca\textsuperscript{2+}] in skinned rat and toad muscles (35). These findings suggest that increasing resting [Ca\textsuperscript{2+}], may play a role in structural and functional muscle defects, particularly after EC. In our study, the changes in immunostaining of desmin, dystrophin, and titin after EC were largely prevented in Ca\textsuperscript{2+}-free solution. Treatment with zero Ca\textsuperscript{2+} and leupeptin also reduced fibronectin entry into muscle cells, indicating that membrane damage also has a Ca\textsuperscript{2+}/calpain-dependent component. Furthermore, removing extracellular Ca\textsuperscript{2+} after the EC protocol improved the muscle force. Thus these data all support the hypothesis that, after eccentric exercise, intracellular calcium rises, activates calpain, and damages the muscle cytoskeleton.

Which calcium-activated protease causes cytoskeletal damage in stretched muscles? Skeletal muscle fiber contains both the ubiquitous calpains, \( \mu \)-calpain and \( m \)-calpain, as well as a muscle-specific calpain, calpain-3 (4, 17). The protease inhibitor leupeptin inhibits \( m \)-and \( \mu \)-calpain (17) and also inhibits calpain-3 once the Ca\textsuperscript{2+}-dependent autolysis process is activated (12, 27). In this study, sustained application of leupeptin largely prevented the changes in immunolabeling for desmin, dystrophin, and titin, suggesting a direct link between cytoskeletal damage and increased calpain activity. It is proposed that calpain-3 might be involved in this process because of its very high sensitivity to a small rise in [Ca\textsuperscript{2+}], even in the nanomolar range (35). Although [Ca\textsuperscript{2+}] needs to be relatively high (\( \geq 2 \mu M \)) for \( \geq 1 \) min to activate detectable levels of \( \mu \)-calpain proteolysis (26), sensitivity increases, as with calpain-3, after its initial activation. Thus, a role for \( \mu \)-calpain in stretch-induced cytoskeletal damage cannot be excluded. Distinguishing between the roles of the various calpains present in muscle will require more selective calpain inhibitors or calpain-knockout mice.

Time course of calpain activation and cytoskeletal protein damage. The time course of activation of calpains following stretch-induced muscle injury also requires further investigation. In our previous studies (2, 37), resting [Ca\textsuperscript{2+}] was only significantly increased at 10 min after stretch. Activation of calpain is potentially fast, as in skinned fibers, in which [Ca\textsuperscript{2+}], can be raised instantly, calpain activity was detectable after 1–2 min after an increase in [Ca\textsuperscript{2+}], (35). However, in situ measurements of calpain-3 activity in human muscle biopsies showed that calpain-3 activation after eccentric exercise was increased but not until 24 h after the ECs (25). Thus it appears surprising that in the present experiments we were able to detect the consequences of Ca\textsuperscript{2+} activation of calpain after only 5 min. There are several possible explanations for this discrepancy. One possibility is that the Ca\textsuperscript{2+} rise occurs very rapidly in restricted regions around the Ca\textsuperscript{2+} influx channels and might not have been detected in the above experiments. Similarly, the detection of calpain-3 activation in muscle biopsies may require homogeneous activation, whereas localized activation may occur much earlier but be difficult to detect. Another possibility is that the loss or disruption of desmin and dystrophin has a mechanical origin although this must be modulated in some way by Ca\textsuperscript{2+}/calpain to be consistent with our results. Finally the difference of time course of Ca\textsuperscript{2+} elevation, activation of calpain, and loss of desmin could result from variations in the experimental approaches, such as species (Balb/c, C57, mdx mouse, rat, toad, and human), degree of muscle activation, or other details of the experimental approach. Role of cytoskeletal proteins in the reduced force following eccentric contractions. The mechanism of the reduced force after EC has long been of interest, and it is often suggested that cytoskeletal damage is one component. Two relevant observations in the present study are 1) that force fell to 76%, 1 min after EC, at a time when no changes in cytoskeletal proteins were detectable; and 2) when cytoskeletal damage was largely eliminated by either zero Ca\textsuperscript{2+} or leupeptin, force, instead of falling to 56% after 1 h, declined to only 69–76%. These findings suggest that the role of the cytoskeletal proteins in force transmission and in maintaining structural integrity required for force production are relatively modest at least in the short term. This agrees with the desmin knockout data where desmin knockout muscles generate lower stress and are less vulnerable to injury compared with wild-type muscles (30). Instead, other mechanisms of force reduction, such as reduced sarcoplasmic reticulum Ca\textsuperscript{2+} release or reduced Ca\textsuperscript{2+}-sensitivity of the contractile proteins or overstretched sarcomeres, are probably more important (2, 24).

In conclusion, this study demonstrates clearly that calpain, a Ca\textsuperscript{2+}-activated protease, is involved in early damage to muscle cytoskeleton after EC. Removal of calcium or application of leupeptin largely prevented the eccentric damage to muscle cytoskeleton but had only moderate effects on the muscle force. These results suggest two potential therapeutic targets for reducing cytoskeletal damage after EC: 1) by lowering Ca\textsuperscript{2+} entry (e.g., stretch-activated channel blockers), or 2) by preventing the activation of proteases.

GRANTS

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