Mechanisms of exercise-induced muscle damage and fatigue: Intracellular calcium accumulation

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Abstract Contraction-induced compromise of muscle function and, in the extreme, muscle damage has been linked to loss of Ca\(^{2+}\) homeostasis and resultant sustained elevation of intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]). Against a background of in vitro approaches, a novel in vivo model permits investigation of the impact of different contraction types (e.g., isometric, ISO; eccentric, ECC) on [Ca\(^{2+}\)] accumulation profiles. [Ca\(^{2+}\)] elevation of ECC-contracted muscle is more rapid and greater in magnitude compared to ISO. Stretch-activated channels (SAC) are responsible, in large part, for this ECC contractions-induced [Ca\(^{2+}\)] elevation. Transient Ca\(^{2+}\) accumulation in the cytosol incurs loss of force production, whereas continuous high levels of [Ca\(^{2+}\)], especially following ECC contractions, lead to muscle damage, including disrupted sarcomeres and membranes, and proceed, subsequently, to muscle regeneration via apoptosis and necrosis.

Keywords: Ca\(^{2+}\) homeostasis, isometric contraction, eccentric contraction, stretch-activated channels, muscle regeneration, apoptosis

Introduction

Calcium ions play a fundamental role in myriad cellular functions. Paramount among these, excitation contraction coupling is regulated by changes in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]). When muscle contracts, there is a transient elevation of [Ca\(^{2+}\)], that helps trigger muscle contraction and thus tension development. In non-fatigued and non-damaged muscle fibers, as the muscle relaxes following contraction, [Ca\(^{2+}\)] decreases immediately. However, repeated contractions induce muscle fatigue (often assessed as the reduction of tetanic force), muscle fiber damage, and prolonged elevation of [Ca\(^{2+}\)], after contraction(s)\(^{1,2}\). Here, as illustrated in Fig. 1, we present two different patterns of muscle contraction-induced intrafiber calcium elevation, and address how alterations in [Ca\(^{2+}\)] accumulation are linked mechanistically to a decline in muscle function. Muscle performance deficits, that are reversible within hours, are considered as muscle fatigue, whereas more prolonged absence of recovery - usually associated with profound structural alterations - is defined as muscle damage. We, therefore, consider the role of skeletal muscle [Ca\(^{2+}\)], and perturbations thereof as critical for acute and chronic functional and dysfunctional signaling processes including apoptosis/necrosis and remodeling.

Intracellular Ca\(^{2+}\) after muscle contractions

Healthy resting myocytes maintain [Ca\(^{2+}\)], under 100 nM\(^{3}\). Myocyte contraction is evoked by the transient elevation of [Ca\(^{2+}\)], which, under normal (i.e., non-fatigued, non-damaged) conditions is almost immediately returned to basal resting levels. Resting [Ca\(^{2+}\)], is increased during long-term contraction. The fatigue protocol (100 Hz tetanic contractions) caused resting [Ca\(^{2+}\)], to increase (pre-condition: 54 nM, post-condition: 132 nM) in mouse single muscle fibers\(^{4}\). However, the degree of increase in [Ca\(^{2+}\)], was far smaller than that consequent to ECC contraction. Importantly, in mouse skeletal muscle, [Ca\(^{2+}\)], may undergo prolonged elevations increasing 2-3 fold above resting levels for up to 48 hours following a bout of ECC contraction\(^{5}\). It is well known this type of contraction causes severe skeletal muscle damage\(^{6,7}\). The connection between altered [Ca\(^{2+}\)], and muscle damage following muscle contractions is well established. The first study documenting elevated [Ca\(^{2+}\)], in muscle, as a consequence of ECC exercise, used a downhill walking protocol in rats, and found that immediately after exercise, mitochondrial [Ca\(^{2+}\)] increased ~3-fold\(^{5}\). More recently, our labo-
ery has demonstrated that myocyte [Ca$^{2+}$] increased, following ECC contractions, using a novel in vivo skeletal muscle (anesthetized rat spinotrapezius) model (Fig. 2) and measuring Ca$^{2+}$-dependent fluorescence intensity in real time. In the case of ECC contractions, the time course about the removal process of Ca$^{2+}$ remains unknown (it is shown as a dashed line). B: There are two major potential sources of Ca$^{2+}$, either SR (Sarcoplasmic reticulum) or the extracellular space. During ISO contractions, Ca$^{2+}$ originates exclusively from the SR. On the other hand, [Ca$^{2+}$], elevation of ECC contractions participates in both stretch-activated channels (SAC) and SR. Mitochondria (Mito) take up Ca$^{2+}$ during an increase in cytoplasmic concentration of Ca$^{2+}$. Continuous high levels of [Ca$^{2+}$], after ECC contractions, lead to muscle damage such as disrupted sarcomeric disruption and membrane damage, and subsequently proceed to muscle regeneration via apoptosis and necrosis.

Thus, ECC contractions induce the loss of [Ca$^{2+}$], homeostasis, and there is substantial evidence that this plays a primary role in skeletal muscle damage. While the precise mechanistic details remain to be elucidated, the pathways for ECC exercise-induced [Ca$^{2+}$] accumulation have been investigated in depth. A classic theory relates to the presence of membrane tearing consequent to mechanical overstretching of the sarcolemma and resultant increased membrane permeability. These sarcolemmal tears permit the influx of intracellular proteins, and influx of large molecules and Ca$^{2+}$ into the sarcoplasm. Although there is support for the presence of mechanical damage following ECC, use of pharmacological Ca$^{2+}$ channel inhibitors to prevent exercise-induced Ca$^{2+}$ accumulation suggests an alternative mechanism. One plausible alternative pathway involves stretch-activated channels (SACs). This latter hypothesis is supported by Allen and colleagues recent demonstration that elevation of intracellular ion levels, after stretched-contractions, was abolished by application of SAC blockers in the healthy skeletal muscle.
Intracellular calcium and muscle damage

Intracellular Ca\(^{2+}\) and calpain. Skeletal muscle fibers contain ubiquitous (μ-calpain and m-calpain, also called calpain-1 and calpain-2) and muscle-specific (calpain-3 also called p94) proteases. In the skeletal muscle, μ-calpain and m-calpain are freely diffusible proteins which bind rapidly to other regulatory proteins when \([\text{Ca}^{2+}]_i\) is increased. For half-maximal proteolytic activity, μ-calpain and m-calpain require ~3–50 μM and ~400–800 μM free Ca\(^{2+}\), respectively\(^{28}\)). The proteolytic activities of μ-calpain and m-calpain are inhibited in a Ca\(^{2+}\)-dependent manner by calpastatin. μ-calpain has many physiological functions such as apoptosis, myogenesis, cell signaling and cell differentiation\(^{29}\)). The ubiquitous calpains play a key role in protein modification through the targeting of the ubiquitin proteasome degradation pathway and play an important role in the repair of the sarcolemma\(^{30}\)).

Calpain-3 is tightly bound in skeletal muscle fibers at the N2A line and M-line regions of the large elastic protein connectin/titin and localizes in the Z-bands\(^{31}\)). The physiological roles of these proteases are not well understood. Calpain-3 seems to be involved in muscle repair and maintenance\(^{32,33}\)). Also it is demonstrated that calpain-3 shifts its location from the M-line to the N2A region when the sarcomere is extended, suggesting that calpain-3 functions as a sarcomere-length sensor, in cooperation with connectin/titin, to mediate signal transduction.

and mdx skeletal muscle\(^{20,23}\)). Similarly, our \emph{in vivo} studies present further evidence that SAC blockers, streptomycin and Gd\(^{3+}\), prevent elevations of \([\text{Ca}^{2+}]_i\), after ECC contractions\(^{30}\).

In marked contrast to ECC, ISO (static) contractions do not obligate and elevate resting \([\text{Ca}^{2+}]_i\), following contractions. Thus, whereas less prolonged, moderate-intensity ISO contractions may produce no detectable increase in resting \([\text{Ca}^{2+}]_i\), in single muscle fibers\(^{22}\), severe chronic ISO stimulation induces gradual \([\text{Ca}^{2+}]_i\), accumulation, and may be associated with muscle fatigue\(^{2,23}\)). Differing from smooth muscle and cardiomyocytes, the influx of extracellular Ca\(^{2+}\), across the sarcolemma, does not contribute significantly to excitation–contraction coupling in skeletal muscle, at least not during acute activation. However, the long-term maintenance of intracellular Ca\(^{2+}\) homeostasis requires Ca\(^{2+}\) influx from the extracellular space, as documented recently for isolated mouse soleus muscles\(^{24}\)). A further consideration is that a mechanical action-independent influx of Ca\(^{2+}\) may exist in myocytes. For instance, insulin-like growth factor (IGF-1) activated Ca\(^{2+}\) permeable channels may contribute to \([\text{Ca}^{2+}]_i\), levels\(^{25,26}\)). In addition, adiponectin, which is an anti-diabetic adipokine, could induce Ca\(^{2+}\) entry into the myocytes via a receptor-dependent pathway\(^{27}\)). A final, but very controversial, consideration is the possibility that there is exercise-associated humoral regulation of Ca\(^{2+}\) accumulation during or after dynamic exercise.

\section*{Intracellular Ca\(^{2+}\) and muscle damage}

\subsection*{Intracellular Ca\(^{2+}\) and calpain.} Skeletal muscle fibers contain ubiquitous (μ-calpain and m-calpain, also called calpain-1 and calpain-2) and muscle-specific (calpain-3 also called p94) proteases. In the skeletal muscle, μ-calpain and m-calpain are freely diffusible proteins which bind rapidly to other regulatory proteins when \([\text{Ca}^{2+}]_i\) is increased. For half-maximal proteolytic activity, μ-calpain and m-calpain require ~3–50 μM and ~400–800 μM free Ca\(^{2+}\), respectively\(^{28}\)). The proteolytic activities of μ-calpain and m-calpain are inhibited in a Ca\(^{2+}\)-dependent manner by calpastatin. μ-calpain has many physiological functions such as apoptosis, myogenesis, cell signaling and cell differentiation\(^{29}\)). The ubiquitous calpains play a key role in protein modification through the targeting of the ubiquitin proteasome degradation pathway and play an important role in the repair of the sarcolemma\(^{30}\)).

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**Fig. 2** Schematic diagram showing direct microscopic observation for spinotrapezius muscles and \([\text{Ca}^{2+}]_i\), \emph{in vivo} \(^{10}\). Fluorescence images were captured from the same area of muscle tissue. Ratio (R: F\(_{340}/F_{380}\)) image was converted and mean gray-scale value measured. ECC contractions were elicited by electrical stimulation (100 Hz) during synchronized muscle stretch of 10% resting muscle length via motor device. This model allows observation of the supply of blood flow and various substrates in a physiological environment.
in response to external stress. Recent studies indicate that calpain-3 protease activity is essential for the signal-transduction pathway by which muscles adapt to physical stress. Thus, whereas in human vastus lateralis muscle, exhaustive sprint cycling does not acutely increase the amount of activated calpain-3 or μ-calpain, nor does ECC knee extensor exercise elevate autolyzed calpain-3 within 3 hours post-exercise; calpain-3 levels increased markedly at 24 hours post-exercise. These results suggest that altered autolysis of calpain-3 may be the consequence of a prolonged rise in resting [Ca²⁺].

Mechanical overload, produced by ECC contractions, induces muscle damage, which leads to myofiber necrosis accompanied with ultrastructural collapse, edema and inflammation of myofibers. These inflammatory reactions achieve their peak responses in 3 days after ECC contractions, and, subsequently, muscle fiber converts to the process of regeneration in ~7 days. After ECC contractions, inflammatory responses progress over the first 3 days following insult, and damaged cells undergo phagocytosis. Given that μ-calpain and calpain-3 are activated after ECC contractions, activated calpains might be associated with the occurrence of muscle damage.

Many researchers have focused on the relationship between [Ca²⁺], and muscle damage. While calpain structure is now well characterized, the activation model and regulation of calpain activity are still unclear. The extent of [Ca²⁺] required to activate μ- and m-calpains and calpain-3 has been clearly identified in vitro. However, it remains to be determined how these relate to [Ca²⁺], in vivo under physiological conditions.

**Calpain and Apoptosis.** Apoptosis is a physiological, highly conserved program of cellular suicide critical for normal development and tissue homeostasis. Apoptosis plays an important physiological role during embryonic development and in the control of cell number in proliferative tissues. Apoptosis is characterized by nuclear condensation, DNA fragmentation and release of mitochondrial cytochrome c into the cytoplasm. The apoptotic program is executed by a cascade of highly specific caspases.

Apoptotic signaling cascades are triggered through several pathways. The mitochondrial pathway can be initiated via intracellular damage or stress signals resulting in the release of cytochrome c from the mitochondria into the cytosol, apoptosis formation, and caspase-9 activation. The endoplasmic reticulum (ER) stress pathway can be initiated by an accumulation of mis-folded and/or unfolded proteins in the ER that can lead to ER stress, Ca²⁺ release, calpain activation, and subsequent caspase-12 and caspase-9 activation.

When [Ca²⁺] increases, m-calpain (or caspase-7)-dependent activation of caspase-12 causes apoptosis via an executioner caspase. In parallel, increasing [Ca²⁺], levels induce calpain-dependent activation of proteins involved in mitochondrial membrane permeability (Bcl-2 interacting protein [Bid], Bcl-2 associated X protein [Bax]). As a result, and dependent on the relative level of B-cell CELL/Lymphoma 2 (Bcl-2) regulators, pro-apoptotic factors are released from the mitochondria. The proteins derived from mitochondria are potential calpain substrates.

Depending on the precise signal received by the cell, calpains will behave as either pro- or anti-apoptotic factors. For example, calpain-dependent hydrolysis of caspase-12, caspase-3, Bid, Bax, Bcl-2 and the appropriately named apoptosis-inducing protein (AIF) have been found to induce apoptosis while cleavage of caspase-3, -7, -8, -9 by the proteases has anti-apoptotic consequences. Due to the broad involvement of calpain activity in the regulation of physiological processes, any alteration of this proteolytic system has the clear potential to promote severe dysfunction.

Sudo and Kano investigated the time course and spatial resolution of ECC-induced myofiber apoptosis histologically. Given that TUNEL-positive nuclei were detected in fibers deprived of dystrophin or sarcoglycan sarcolemmal staining, following 6 and 24 hours of ECC contractions, the loss of several membrane skeletal proteins after ECC contractions could lead to fiber death by apoptosis or necrosis.

It has been proposed that calpain-3 plays a role in muscle repair and maintenance, myogenesis and apoptosis. Sudo and Kano reported that, in myofiber longitudinal sections, myonuclear apoptosis occurs at the subsarcolemmal level, as opposed to the central cytoplasm, at 7 and 14 days after ECC contractions. Furthermore, Stuelsatz et al. reported that calpain-3 participates in the establishment of a pool of reserve cells, by decreasing the transcriptional activity of the key myogenic regulator MyoD - a process that occurs via proteolysis, independent of the ubiquitin-proteasome degradation pathway. Their results identified calpain-3 as a potential new player in the muscular regeneration process by promoting renewal of the satellite cell compartment.

Different exercise modes result in the activation of different muscle fiber types. In rodent skeletal muscle, expression of μ-calpain is not dependent on muscle fiber type. Recently, however, McMillan and Quadrilatero determined that caspase-3, -8, and -9 activity, calpain activity, and number of apoptotic cells were also significantly higher in the red compared with white gastrocnemius. In contrast, Murphy and Lamb have shown that calpain-3 activity was ~1.9 times higher in individual muscle fiber segments from extensor digitorum longus (EDL) muscle compared to soleus muscle. Further studies are needed to clarify whether differences in muscle fiber type influences the association between calpain activation and exercise-induced myofiber apoptosis.

The current data suggest that exercise-induced calpain...
activation plays a role in regulating skeletal muscle apoptosis. However, there are still several unsolved issues that need to be addressed - paramount among which is unveiling of the relationship between exercise-induced calpain activation and myofiber apoptosis in skeletal muscle.

Intracellular Ca\textsuperscript{2+} and hypertrophic response

Increase in [Ca\textsuperscript{2+}], levels not only promotes proteolysis but is also responsible for protein synthesis through activation of downstream transcriptional pathways. It is well known that two major routes for skeletal muscle development and growth are the insulin-like growth factor 1 (IGF-I) and Ca\textsuperscript{2+}/calmodulin pathways. IGF-1 is a potent stimulating factor of myofibrillar protein synthesis via Mammalian target of rapamycin (mTOR) pathway, mitogen-activated protein kinase (MAPK) and calcineurin pathway\textsuperscript{69}). The mechanism about mTOR and MAPK signal transduction is summarized in recent reviews\textsuperscript{68}). Here we focus on understanding the Ca\textsuperscript{2+}/calmodulin-dependent transcriptional pathways. Calmodulin is a multifunctional signal transducer that undergoes conformational changes before activating a wide range of binding substrates; mainly downstream phosphatases (calcineurin) and kinases (CaMKs)\textsuperscript{69}). Changes in [Ca\textsuperscript{2+}], regulate transcriptional activation through the calcineurin/CaMK pathways. In turn, these trigger the signaling pathway that ultimately results in muscle growth and hypertrophy\textsuperscript{25,70}). Activated calcineurin dephosphorylates the nuclear factor of activated T-cell (NFAT) resulting in translocation of NFAT from the cytoplasm to the nucleus. As a result, it activates specific transcriptional factors such as myocyte enhancer factor 2 (MEF2) and GATA4/2 which induces the expression of hypertrophic genes such as βmyosin, αactin, Glut4 and utrophin A. In contrast, activated calcineurin inhibits the expression of myostatin. The mRNA and protein levels of myostatin were significantly increased in mice treated with a calcineurin inhibitor (cyclosporine A)\textsuperscript{71}).

Intracellular Ca\textsuperscript{2+} and muscle fatigue

As mentioned above, skeletal muscle fatigue may be defined with respect to a progressive decline of muscle force production or power output during and following prolonged or repeated muscle contractions. Resolution of the mechanisms responsible (for fatigue) has been challenging, in part, due to the multivariate nature of the fatigue process, the complexity of involved pathways and the influence of, and often lack of control of, intracellular perturbations (e.g. depletion of energy substrates, accumulation of metabolic by-products that impair the contractile process and/or central fatigue). Therefore, to provide insights into the mechanisms of muscle fatigue, many studies have been performed on isolated whole muscles or single fibers as an expedient to permit greater control over the experimental environment.

Force development in skeletal muscle is dependent on Ca\textsuperscript{2+} release from the sarcoplasmic reticulum (SR) during excitation-contraction (EC) coupling. In classical studies, Eberstain and Shadow\textsuperscript{20}) showed the beneficial effect of caffeine which enhanced force development in fatigued muscle fibers via increased SR Ca\textsuperscript{2+} release. They proposed that the impairment of Ca\textsuperscript{2+} release from the SR is a key factor in fatigue development. This phenomenon confirmed many studies using isolated single muscle fibers, and showed that application of caffeine, to a muscle fatigued with repetitive tetanic stimulation, almost completely restored the force development by improving Ca\textsuperscript{2+} release from the SR to pre-fatigue levels\textsuperscript{70}). More recent studies have demonstrated that SR Ca\textsuperscript{2+}-sequestering ability is impaired after high intensity exercise (maximal O\textsubscript{2} uptake, \textit{VO}_\textsubscript{max})\textsuperscript{71}). Thus, it is generally accepted that limitations of SR Ca\textsuperscript{2+} handling (i.e. Ca\textsuperscript{2+} release and uptake) can represent an important component in the etiology of muscle fatigue.

Although those mechanisms underlying impaired SR Ca\textsuperscript{2+} regulatory functions in fatigued skeletal muscle have not been completely elucidated, there is evidence that metabolic perturbations (e.g. ADP, H\textsuperscript{+}, Pi, ROS) within the cell affect [Ca\textsuperscript{2+}]\textsuperscript{24,74-76}). For example, Fryer et al.\textsuperscript{73}) demonstrated the possibility of a Ca-Pi precipitation-induced reduction of the amount of Ca\textsuperscript{2+} stored in SR. More recently, Dutuka et al., using the intact EC coupling skinned fiber preparation\textsuperscript{78}), reported that elevated [Pi] reduced the action potential-induced SR Ca\textsuperscript{2+} release.

Our recent study has demonstrated that the peak level of [Ca\textsuperscript{2+}], is increased by 21.5% after 10 sets of contractions (i.e. total 500 tetanic ISO contractions, 42.8% after ECC contractions) in the in vivo spinotrapezius muscle preparation\textsuperscript{9}). The observed rise, in resting [Ca\textsuperscript{2+}], during muscle fatigue, was also seen in isolated toad muscle\textsuperscript{70}) and mouse single muscle fiber preparation using a low frequency fatigue protocol\textsuperscript{4,80}). [Ca\textsuperscript{2+}], accumulation may result from attenuation of SR Ca\textsuperscript{2+} uptake and/or leak from the SR and/or extracellular space. Matsunaga et al.\textsuperscript{80}) demonstrated the increased content of carbonyl groups in SR Ca\textsuperscript{2+}-ATPase, after high intensity (\textit{VO}_\textsubscript{2max}) exercise, and proposed that oxidation of SR Ca\textsuperscript{2+}-ATPase contributes to impairment of the SR Ca\textsuperscript{2+} handling and repair of oxidized proteins during recovery. Lamb et al.\textsuperscript{81}) demonstrated that increased [Ca\textsuperscript{2+}], in fibers, exposed to high [Ca\textsuperscript{2+}] solution, distorted or severed triad junctions and produced Z-line aberrations in skinned fibers of rat and toad. This concept was supported by Chin et al.\textsuperscript{82}) who argued that the “Ca\textsuperscript{2+}-time integral” (i.e., [Ca\textsuperscript{2+}], x exposure time) is most critical in determining the magnitude of the SR Ca\textsuperscript{2+} release deficit.

Although insightful, these investigations, using isolated single or skinned fiber (i.e. in vitro studies), also bring inherent limitations, especially in relating the data obtained to in vivo physiological control. For instance, as distinct from in vivo conditions: 1. Single fibers do not survive...
well at physiological temperatures, and are often maintained at hypothermic temperatures. 2. There is no blood circulation which negates normal O2 and substrate supply. 3. Non-physiologic high O2 environments are often created. 4. There are no interactions among different muscle fiber types. 5. Neural modulation is abolished. 6. The intracellular ion dynamics that may affect muscle fatigue are impacted. All of these impositions will act to disrupt the in vivo physiology via perturbations of intracellular redox, metabolic control processes and a host of intracellular signaling mechanisms. Pursuant to this consideration, and to more closely approach physiological conditions, Allen et al. 16 investigated intracellular phosphate concentration ([P(i)]), measured by (31)P nuclear magnetic resonance (NMR) and myoplasmic and sarcoplasmic reticulum (SR) calcium concentrations during tetanic contractions, in vivo mouse skeletal muscle. They showed that the initial phase of muscle fatigue is accompanied by a rise in [P(i)] and a decrease in tetanic myoplasmic calcium – a very different scenario from that observed in vitro 24,76,83,84. Thus, a compelling case may be made for studying the mechanisms of muscle fatigue in vivo using contemporary models as and when they become available.

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