The Role of Ca^{2+} in Muscle Cell Damage

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ABSTRACT: Skeletal muscle is the largest single organ of the body. Skeletal muscle damage may lead to loss of muscle function, and widespread muscle damage may have serious systemic implications due to leakage of intracellular constituents to the circulation. Ca^{2+} acts as a second messenger in all muscle and may activate a whole range of processes ranging from activation of contraction to degradation of the muscle cell. It is therefore of vital importance for the muscle cell to control $[\text{Ca}^{2+}]$ in the cytoplasm ($[\text{Ca}^{2+}]_c$). If the permeability of the sarcolemma for Ca^{2+} is increased, the muscle cell may suffer Ca^{2+} overload, defined as an inability to control $[\text{Ca}^{2+}]_c$. This could lead to the activation of calpains, resulting in proteolysis of cellular constituents, activation of phospholipase A_2 (PLA_2), affecting membrane integrity, an increased production of reactive oxygen species (ROS), causing lipid peroxidation, and possibly mitochondrial Ca^{2+} overload, all of which may further worsen the damage in a self-reinforcing process. An increased influx of Ca^{2+} leading to Ca^{2+} overload in muscle may occur in a range of situations such as exercise, mechanical and electrical trauma, prolonged ischemia, Duchenne muscular dystrophy, and cachexia. Counteractions include membrane stabilizing agents, Ca^{2+} channel blockers, calpain inhibitors, PLA_2 inhibitors, and ROS scavengers.

KEYWORDS: calpain; phospholipase A_2 ; reactive oxygen species; exercise; Duchenne muscular dystrophy; surfactant; skeletal muscle

INTRODUCTION

Skeletal muscle is the largest single organ of the body, constituting about 40% of the mass of the average human body. Skeletal muscle is formed of long multinucleate, cylindrical cells called muscle fibers. Muscle damage may lead to loss of function, and widespread damage to muscle may have serious systemic implications due to leakage of intracellular constituents to the circulation.

Ca^{2+} plays a very important role in skeletal muscle. All muscles use Ca^{2+} as their main regulatory and signaling molecule, and the function of all muscle types is controlled by Ca^{2+} as a second messenger.

In this review I will focus on the role of Ca^{2+} in the healthy muscle cell and in the development of skeletal muscle damage. I will present a model placing an increased influx of Ca^{2+} across the cellular membrane as the initiating factor leading to skele-

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tal muscle damage and propose a range of clinical situations where this may be the case. Finally I will evaluate possible countermeasures to prevent or reduce skeletal muscle damage.

Ca^{2+} IN THE HEALTHY MUSCLE CELL

The concentration of free Ca^{2+} in the cytosol ($[\text{Ca}^{2+}]_c$) is maintained at ~ 50 nM in the resting state.¹ This is in sharp contrast to the concentration of Ca^{2+} in the extracellular fluid ($[\text{Ca}^{2+}]_o$), which is around 1 mM, creating an enormous chemical gradient for Ca^{2+} across the cellular membrane. Despite a very low permeability of the sarcolemma for Ca^{2+} there is continuous passive diffusion of Ca^{2+} into the muscle cells owing to the large electrochemical gradient across the membrane.² In order to maintain the low resting $[\text{Ca}^{2+}]_c$ the muscle cell must actively remove Ca^{2+} from the cytosol by the expenditure of ATP using the Ca^{2+} -ATPases in the sarcoplasmic reticulum (SR) and sarcolemmal/T-tubular membranes.²⁻⁴ During a contraction relaxation cycle there is a large flux of Ca^{2+} ions from the SR to the contractile filaments and back, causing relatively large (up to 100-fold locally) but short-lived changes in $[\text{Ca}^{2+}]_c$. Excitation is also associated with a small influx of Ca^{2+} across the cellular membrane (FIG. 1).⁵

FIGURE 1. Schematic diagram of a skeletal muscle cell showing the fluxes of Ca^{2+} during excitation. As the action potential moves along the cellular membrane and down into the T-tubules, the electric signal is transferred to the SR, where it is converted to a chemical signal by the release of Ca^{2+} . The increase in $[\text{Ca}^{2+}]_c$ activates contraction. The Ca^{2+} released from the SR is quickly reaccumulated into the SR by Ca^{2+} -ATPases in the SR membrane and contraction is stopped. Thus during contraction there is a large flux of Ca^{2+} from the SR to the contractile filaments and back. The major storage compartment for Ca^{2+} is the SR, although the mitochondria may also participate in Ca^{2+} regulation. Ca^{2+} influx can also occur through Ca^{2+} -conducting ion channels and Ca^{2+} is exported from the cell via the Ca^{2+} -ATPase in the cellular/T-tubular membrane or via $\text{Na}^+/\text{Ca}^{2+}$ exchange. (1) SR Ca^{2+} -ATPase; (2) SL Ca^{2+} -ATPase; (3) $\text{Na}^+/\text{Ca}^{2+}$ exchanger.

In the resting cell, approximately 5% of the total energy turnover is dedicated to Ca^{2+} cycling, whereas during contraction Ca^{2+} cycling may consume 20–50% of the total energy turnover.⁶

Transport Mechanisms

The skeletal muscle cell has several ways of clearing Ca^{2+} from the cytoplasm. The most important is the SR Ca^{2+} -ATPase, which is responsible for reuptake of Ca^{2+} into the SR. It has a high affinity for Ca^{2+} ($K_m < 0.5 \mu\text{M}$) on the cytoplasmic side and a maximum velocity of 6.4 and $2.4 \mu\text{mol/g}$ fiber protein/min in fast and slow single human fibers, respectively.⁷ The sarcolemmal and T-tubular Ca^{2+} -ATPases (SL Ca^{2+} -ATPase) actively export Ca^{2+} from the muscle cell. These are high-affinity ($K_m = 0.5 \mu\text{M}$) but low-capacity systems ($12\text{--}30 \text{ nmol } \text{Ca}^{2+}/\text{mg}$ protein/min in rabbit white muscle)^{2,3} that are active in regulating $[\text{Ca}^{2+}]_c$ especially under resting conditions.^{2,8}

A second export mechanism is Na^+ - Ca^{2+} exchange. Na^+ - Ca^{2+} exchange has been widely studied in cardiac muscle, where it plays a major role in Ca^{2+} regulation. In skeletal muscle the capacity and quantitative importance of Na^+ - Ca^{2+} exchange is more controversial. The existence of Na^+ - Ca^{2+} exchange in mammalian skeletal muscle has been documented in several studies.^{9,10} It is a low-affinity but high capacity system. In sarcolemmal vesicles the transport rate was 5–10-fold lower and K_m for Ca^{2+} was an order of magnitude higher in $\text{Na}^+/\text{Ca}^{2+}$ exchange compared to Ca^{2+} -ATPase transport.^{3,11} Studies on frog muscle and single mouse fibers showed that $\text{Na}^+/\text{Ca}^{2+}$ exchange activity was low during normal resting conditions and only becomes measurable when $[\text{Ca}^{2+}]_c$ is increased substantially above 80 nM.¹⁰ Thus it was suggested that Na^+ - Ca^{2+} exchange may be active in Ca^{2+} removal at high $[\text{Ca}^{2+}]_c$, as during fatiguing stimulation,¹⁰ and may participate in the long-term regulation of myoplasmic Ca^{2+} .⁹

Internal Storage of Ca^{2+}

The muscle cell is capable of storing large amounts of Ca^{2+} . Two organelles are involved in Ca^{2+} storage: the sarcoplasmic reticulum (SR) and the mitochondrion.

The Sarcoplasmic Reticulum (SR)

The major storage compartment of the muscle cell is the SR, which is traditionally considered the organelle responsible for the ambiguous regulation of Ca^{2+} . Because of the presence of the Ca^{2+} -binding protein calsequestrin, the buffer capacity of the SR for Ca^{2+} is large. Calsequestrin is capable of binding 40–50 moles of $\text{Ca}^{2+}/\text{mol}$.¹ The total concentration of Ca^{2+} within the SR at rest has been reported to be 11 and 21 mM for slow-twitch and fast-twitch fibers, respectively, whereas the concentration of free Ca^{2+} is kept around 1 mM.¹² Studies have shown that at resting $[\text{Ca}^{2+}]_c$ the SR of slow-twitch fibers is saturated with Ca^{2+} , whereas the SR of fast twitch fibers is only one-third saturated.¹² This means that the buffering capacity of the SR in slow-twitch fibers is smaller than that of the fast-twitch fibers.

Mitochondria

As early as the 1960s, it was discovered that the mitochondria were capable of taking up large amounts of Ca^{2+} .¹³ In mitochondria Ca^{2+} uptake occurs via the Ca^{2+}

uniporter located in the inner membrane of the mitochondrion. The K_m for the uniporter is between 10 and 100 μM .¹⁴ Since global increases of $[\text{Ca}^{2+}]_c$ to this range are usually only seen under pathologic conditions it was seriously questioned whether Ca^{2+} was taken up by the mitochondria under physiological conditions. Recently the mitochondria have reemerged as active players in the regulation of Ca^{2+} . On the basis of 3-D fluorescence imaging, it has been proposed that the mitochondria are located close to Ca^{2+} -release channels of the SR, and upon activation the mitochondria sense highly localized increases in $[\text{Ca}^{2+}]$ (20–30 μM) as it is being released. The high local $[\text{Ca}^{2+}]$ enables the uniporter to take up Ca^{2+} .¹⁵ Recently it was shown *in vivo* in mouse skeletal muscle that mitochondria take up Ca^{2+} during contraction and release it during relaxation. The mitochondrial Ca^{2+} uptake was delayed by a few milliseconds compared with the cytosolic rise and occurred both during a single twitch and upon tetanic contraction.¹⁶ Thus, it has become apparent that the mitochondria may play an important role in the regulation of Ca^{2+} in skeletal muscle.

EFFECTS OF Ca^{2+} OVERLOAD

Ca^{2+} overload, defined as an excessive accumulation of Ca^{2+} in the muscle cell, resulting in an inability of the muscle cell to control $[\text{Ca}^{2+}]_c$, is a serious condition involved in a wide range of situations leading to muscle cell damage.

As mentioned previously, Ca^{2+} is an important messenger in skeletal muscle, capable of activating a wide range of processes. Therefore, it is vital to the muscle cell to tightly control $[\text{Ca}^{2+}]_c$, a function performed by the combined action of the SR, the mitochondria, and the SL Ca^{2+} -ATPase. In addition fast muscle fibers from lower vertebrates and small mammals contain high amounts of the cytosolic Ca^{2+} -binding protein parvalbumin.¹⁷ In humans parvalbumin is only present in the intrafusal fibers and is therefore not important in Ca^{2+} regulation in skeletal muscle.¹⁸

If the permeability of the sarcolemma for Ca^{2+} is increased (e.g., due to damage or opening of Ca^{2+} -conducting channels), a larger influx of Ca^{2+} will occur, increasing the Ca^{2+} load on the cell. Because of the large storage capacity of the SR and the mitochondria this may not necessarily present a problem for the cell, as the extra Ca^{2+} can be stored. However, if the increased influx of Ca^{2+} persists, Ca^{2+} overload of the cell may result.

Increases in $[\text{Ca}^{2+}]_c$ may result in activation of Ca^{2+} -activated neutral proteases (calpains), activation of PLA_2 , an increased production of ROS, and/or mitochondrial Ca^{2+} overload. Each of these will be discussed in the following sections.

Calpain

It has become increasingly clear over the past years that calpains play an important regulatory role in cellular functions. Three types of calpains are of interest in skeletal muscle: calpain 1 (μ -calpain), calpain 2 (m-calpain), and calpain 3 (p94). The latter is almost exclusively expressed in skeletal muscle¹⁹ and the amount of mRNA for this calpain is 10 times higher than mRNA for calpains 1 and 2.²⁰

The three calpains differ in their sensitivity to Ca^{2+} . The threshold for initial activation is lowest for calpain 3 (approximately 0.5 μM),²¹ while for calpain 1 it is

3 μM and for calpain 2 it is 400 μM . However, when the protein is autolyzed, proteolytic activity occurs at $[\text{Ca}^{2+}]$ of 0.5–2 μM for calpain 1 and 50–150 μM for calpain 2.¹⁹ Recent evidence suggests that autolysis is necessary for activation of calpain 3.²²

Calpains 1 and 3 are expected to be activated at physiological $[\text{Ca}^{2+}]$ and are thought to be important in the regulatory function of the cell. Calpain 2, with the higher Ca^{2+} requirement, is thought to be involved in fiber degeneration.²³

Calpain cleaves a variety of protein substrates including cytoskeletal, myofibrillar, and membrane proteins. In particular titin (α -connectin), a large elastic protein that tethers the thick filament at the center of the sarcomere, by anchoring it to the Z-line, is readily proteolyzed by calpain.¹⁹ Calpain is closely associated with the I and the Z band regions and calpain-mediated degradation is thought to contribute to the changes in muscle structure (Z-line streaming) and function (EC uncoupling) that occur immediately after exercise.^{24–26} Increases in calpain activity in muscle has been observed during prolonged running,^{26,27} following chronic low-frequency stimulation,²³ in sepsis²⁸ and in muscle from *mdx* mice (the murine model for Duchennes muscular dystrophy).²⁹

PLA₂

PLA₂ may be activated by elevated $[\text{Ca}^{2+}]_c$.³⁰ The activated PLA₂ will attack mitochondrial and other membrane phospholipids, giving rise to lysophospholipids and free fatty acids. Lysophospholipids will disrupt membrane lipid organization and free fatty acids may have a detergent action, causing membrane damage.³¹ Among the free fatty acids liberated is arachidonic acid, which may promote ROS production by the mitochondria (see the next section). There is evidence that increased $[\text{Ca}^{2+}]_c$ activates PLA₂, based on the observation that enzyme efflux from experimentally damaged muscles (suffering Ca^{2+} overload) can be blocked by PLA₂ inhibitors.^{31,32} NDGA (a lipo-oxygenase inhibitor) completely inhibited CK efflux from skeletal muscle following treatment with the Ca^{2+} ionophore A23187 or DNP (dinitrophenol, poisoning of the mitochondria) showing that PLA₂ activation, and in particular the lipooxygenase pathway, affects cellular membrane integrity.³³

Reactive Oxygen Species

The mitochondria of skeletal muscle cells continuously generate ROS. Increased Ca^{2+} load on the muscle cell may increase superoxide anion synthesis. Enzymatic synthesis by xanthine oxidase is stimulated by increased $[\text{Ca}^{2+}]_c$.³⁴ Furthermore, it has been shown that mitochondrial H_2O_2 production is stimulated by physiological levels of ADP and calcium, possibly via a PLA₂-mediated pathway.³⁵

Oxidative stress may disrupt the normal Ca^{2+} handling kinetics in muscle cells by disturbing SR function³⁶ and may be a causative factor in opening of the mitochondrial permeability transition pore (mPTP), especially in combination with high mitochondrial Ca^{2+} content ($[\text{Ca}^{2+}]_m$) (see the next section).^{37,38} Oxidative stress leads to lipid peroxidation, affecting membrane structures in the cell, which has been shown following exhaustive exercise in both human and animal studies.^{39–41}

Mitochondrial Ca^{2+} Overload

In the healthy cell, mitochondria may assist in refilling SR Ca^{2+} stores by interaction with store-operated Ca^{2+} channels;⁴² furthermore the mitochondria may act as Ca^{2+} buffers, rapidly taking up surplus Ca^{2+} and slowly releasing it again to avoid detrimental oscillations in $[\text{Ca}^{2+}]_c$. If the muscle cell faces increased influx of Ca^{2+} from the outside, the mitochondria will accumulate Ca^{2+} .

It is by now well established that small increases in mitochondrial Ca^{2+} content ($[\text{Ca}^{2+}]_m$) stimulate ATP synthesis. Uptake of Ca^{2+} during activity may therefore serve as an important signal to ensure that energy production matches energy expenditure. However, larger accumulations may have detrimental effects. As mentioned above, increased Ca^{2+} load of the mitochondria will increase ROS production, and with it, the risk of increasing nonspecific inner membrane permeabilization.⁴³ Increases in $[\text{Ca}^{2+}]_m$ also increase the probability of opening of the permeabilization transition (mPTP) pore.³⁷ The mPTP is a large conductance pore spanning both the inner and outer membrane of the mitochondria. The pore opens most clearly under specific and usually pathologic conditions. Opening leads to depolarization of the mitochondrial membrane potential, effectively shutting down ATP production and resulting in a massive efflux of Ca^{2+} from the mitochondria, causing further rises in $[\text{Ca}^{2+}]_c$ and initiating a vicious cycle leading to apoptosis or necrosis.^{37,38}

The Vicious Cycle

The vicious cycle model describes how the above-mentioned factors act in concert, creating a self-reinforcing cycle that leads to muscle cell necrosis/apoptosis (Fig. 2).

An increase in Ca^{2+} influx across the cellular membrane is observed in many situations (some of which I will discuss in detail later). Influx of Ca^{2+} will lead to local increases in $[\text{Ca}^{2+}]$ in the subsarcolemmal compartments, which may lead to local activation of calpain. Some of the Ca^{2+} will be exported again by the SL Ca^{2+} -ATPase and some will be taken up by the SR via the SR Ca^{2+} -ATPase and stored. The SR is capable of storing quite large quantities of Ca^{2+} . However, buffer capacity differs between different fiber types. The SR of fast-twitch fibers is capable of increasing their Ca^{2+} content threefold, whereas the SR of slow-twitch fibers is almost saturated at resting $[\text{Ca}^{2+}]_c$. Thus in the fast-twitch fibers, the SR represents a large buffer capacity, whereas slow-twitch fibers most likely rely on their more abundant mitochondria for Ca^{2+} buffering. In addition fast fibers from lower vertebrates and small mammals contain high concentrations of parvalbumin,¹ which may act as a significant buffer for Ca^{2+} .

The mitochondria will participate actively in the Ca^{2+} handling, particularly at the time of SR saturation. Mitochondria are capable of storing large amounts of Ca^{2+} ; however, if the storage capacity is exceeded, detrimental consequences may occur. Increased Ca^{2+} load leads to increased production of ROS, causing peroxidation of membrane lipids, and possibly decreases in ATP synthesis. Mitochondrial Ca^{2+} overload also increases the risk of opening of the mPTP, which will ultimately lead to apoptosis or necrosis. Local increases in $[\text{Ca}^{2+}]_c$ may lead to PLA_2 activation, resulting in degradation of the cellular membrane as well as the membranes of organelles. This leads to an increased production of arachidonic acid leading to fur-

FIGURE 2. The “vicious cycle” model of how an initial event or condition leading to an increased influx of Ca²⁺ may result in muscle cell apoptosis or necrosis.

ther increases in production of ROS in the mitochondria. Finally, activation of calpain is expected, leading to degradation of cytoskeletal, myofibrillar, and membrane proteins. These processes ultimately lead to an increased permeability of the cellular membrane, causing further influx of Ca²⁺, and the vicious cycle is activated.

The muscle cell has a high capacity for repairing damages to the membrane,⁴⁴ and therefore if the degradation process is stopped in time, the vicious cycle may be halted and the muscle cell may recover. However, a “point of no return” exists, beyond which it is not possible for the cell to regain control of cellular Ca²⁺, eventually leading to the destruction of the cell.

Ca²⁺ OVERLOAD SCENARIOS

Ca²⁺ overload occurs in a wide range of situations, and even though many of these situations seems to differ, a common denominator across the following examples is an initial increase in Ca²⁺ influx leading to muscle Ca²⁺ overload and muscle damage.

One of the serious consequences of muscle Ca²⁺ overload is rhabdomyolysis, a condition that is characterized by muscle tissue breakdown, resulting in the release of cytosolic components into the blood circulation. The degree of rhabdomyolysis ranges from an asymptomatic illness with elevation in the plasma CK level to a life-threatening condition associated with extreme elevations in plasma CK, electrolyte imbalances, acute renal failure, and disseminated intravascular coagulation.⁴⁵ Hypocalcemia is frequently observed during rhabdomyolysis owing to massive influx of Ca²⁺ into the muscle tissue. Administration of Ca²⁺ in this situation should be very carefully considered as clinical investigations have shown that it may worsen the damage considerably.⁴⁶

Exercise

It is well documented in both human and animal studies that prolonged or unaccustomed exercise, and in particular lengthening (eccentric) exercise, may lead to muscle cell damage.^{47–49} This damage may be ultrastructural alterations leading to loss of force or loss of sarcolemmal integrity leading to loss of intracellular enzymes, such as lactate dehydrogenase (LDH) or CK. Although not necessarily a pathologic phenomenon, it is a frequently occurring consequence of certain types of contractile activity, and might serve as an adaptive response to exercise. However, if the exercise is excessive, rhabdomyolysis may occur.

Much attention has been diverted to the milder forms of muscle damage—in particular, delayed onset muscle damage (DOMS), since it is of great importance for muscle performance in athletes. A model for the development of DOMS was proposed by Armstrong in 1990.⁴⁹ The model describes four stages in exercise-induced muscle injury: (1) the initial stage; (2) the autogenic stage, or the “ Ca^{2+} overload” stage; (3) the phagocytic stage; and (4) the regenerative stage. The autogenic stage is characterized by Ca^{2+} overload. This is supported by evidence that exercise leads to an increased activation of calpain²⁶ as well as PLA_2 ,⁵⁰ both of which are activated by increased $[\text{Ca}^{2+}]_i$. Activation of calpain and PLA_2 may then further worsen the damage as described above in the section on the vicious cycle. The increased production of ROS may also have deleterious effects on muscle cell integrity.

There has been much debate on the nature of the initial event triggering DOMS. Some believe that mechanical damage to the fiber initiates exercise-induced muscle damage.⁴⁸ This may very well be the case during lengthening contractions, where the strain on the muscle fiber is high. Our lab has shown that excitation of rat skeletal muscle both *in situ* and *ex vivo* (isometric and shortening contractions) is associated with an increased influx of Ca^{2+} (up to 34-fold increase within the first 30 sec of stimulation) and that prolonged stimulation may lead to accumulation of Ca^{2+} in the muscle cells and loss of membrane integrity.^{5,51,52} Similar, but smaller accumulations of Ca^{2+} in muscles from athletes after prolonged running has also been reported.^{47,53} We suggest that the excitation-induced influx of Ca^{2+} may act as or contribute to the initiating factor leading to muscle damage during prolonged or otherwise excessive exercise.

Trauma

Trauma affects muscle membrane integrity directly. In this section I will discuss mechanical and electrical trauma both leading to an increased influx of Ca^{2+} although by two very different mechanisms.

Mechanical trauma causes direct injury to the cellular membrane causing Ca^{2+} and Na^+ to flood the injured tissue. Often circulation is compromised, causing ischemia of the tissue, and the damage process may accelerate in the following reperfusion period. The effect is a massive Ca^{2+} overload of the muscle cells, resulting in necrosis of many fibers.⁴⁵

Electrical trauma due to high-voltage electrical injury or lightning may lead to electroporation of cellular membranes, rendering them permeable to ions and molecules. This will result in an increased influx of Ca^{2+} .⁵⁴ If resealing does not occur rapidly enough, cellular Ca^{2+} homeostasis will be lost, leading to destruction of the

muscle cell. (Electric force-mediated membrane disruption will be reviewed elsewhere in this issue by Raphael Lee.)

Ischemia

Prolonged ischemia and reperfusion results in tissue damage and necrosis in many organs.

Prolonged ischemia is associated with hypoxia. Results from this laboratory as well as others have shown that anoxia increases the permeability of the membrane for Ca^{2+} and may lead to a loss of cellular integrity.⁵⁵⁻⁵⁷ The mechanism behind the increased influx of Ca^{2+} has not been identified, but if anoxia was combined with electrical stimulation, possibly depleting cellular ATP stores, the stimulation-induced accumulation of Ca^{2+} was markedly increased and so was the release of LDH.⁵⁵ During prolonged ischemia/anoxia, ATP levels would be expected to be low, possibly compromising the Ca^{2+} handling ability of the muscle cell. In line with this increasing baseline $[\text{Ca}^{2+}]_c$ was observed during fatiguing stimulation under hypoxic conditions.⁵⁸

In vivo reperfusion may be associated with an increased generation of ROS from circulating neutrophils, causing further damage to the cellular membrane.⁵⁹

DMD

The human Duchenne muscular dystrophy (DMD) is the best known of the muscular dystrophies, since it is one of the most frequent genetic human diseases.¹ It is characterized by muscle fiber necrosis and progressive muscle wasting and weakness. Ca^{2+} seems to be critically involved in the pathology of DMD. The primary defect in DMD is the lack of dystrophin, a subsarcolemmal cytoskeletal protein. In normal muscle dystrophin links the cytoskeleton, via a complex of membrane proteins, to laminin in the extracellular matrix mechanically stabilizing the cellular membrane.⁶⁰ Lack of dystrophin renders the sarcolemma more vulnerable to mechanical damage. The number of transient disruptions of the sarcolemma was found to be sixfold higher in muscles from exercised *mdx* mice compared to controls.⁶¹ These transient disruptions may give rise to Ca^{2+} entry, leading to local activation of calpain. Studies have shown that $[\text{Ca}^{2+}]$ in the subsarcolemmal compartments was 4.5-fold higher in *mdx* myotubes following depolarization compared to controls.⁶² Furthermore an increased calpain activity has been observed in muscle from *mdx* mice.²⁹ It was suggested that the activation of calpain would lead to proteolysis of the Ca^{2+} leak channels (possibly TRP channels⁶³), causing an increased activity. This in turn would further increase Ca^{2+} influx and Ca^{2+} -activated proteolysis.⁶⁴ An increased Ca^{2+} leak channel activity has been observed in *mdx* fibers and this was thought to be the mechanism behind the greater levels of Ca^{2+} influx, leading to increased $[\text{Ca}^{2+}]_c$ and increased proteolysis.⁶⁵ In addition to the increased calpain activity an increase in PLA_2 activity was shown in DMD patients.⁶⁶

Muscle Cachexia

Muscle cachexia is a common metabolic response to several disease states seen in surgical patients including sepsis, severe injury, renal failure, and cancer.⁶⁷ Although not identical, the intracellular mechanisms and molecular regulation of mus-

cle atrophy are similar in many of these conditions. The early phase of muscle cachexia may be beneficial, providing amino acids for gluconeogenesis and acute-phase protein synthesis. However, prolonged and severe cachexia has significant deleterious consequences.⁶⁷

It is quite likely that early cachexia is associated with an increased influx of Ca^{2+} . Sepsis has been shown to increase ^{45}Ca uptake and Ca^{2+} content in isolated rat skeletal muscle, which was associated with an increased protein breakdown.^{68,69} Furthermore, muscle cachexia induced by severe injury, sepsis, and cancer is associated with increased gene expression and activity of calpain 1, 2, and 3.⁶⁷ It has been proposed that early cachexia is associated with a calcium-calpain-dependent degradation of Z-disks and release of myofilaments from the myofibrils. This is followed by ubiquitination of the myofilaments and a subsequent degradation by the proteasome.⁶⁷ Supported for this was provided by morphologic evidence showing disintegration of the Z-disk and release of myofilaments from myofibrils in cachectic muscle from septic rats. Treatment with dantrolene (inhibiting the release of calcium from intracellular stores) prevented the sepsis-induced release of myofilaments.⁷⁰

In addition, mitochondria from septic animals demonstrate substantially higher (PLA₂-mediated) H_2O_2 formation compared to controls,³⁵ increasing the oxidative stress on the muscle fiber.

COUNTERACTING MEASURES

Inhibiting Influx of Ca^{2+}

One obvious way of preventing Ca^{2+} -induced muscle damage is to inhibit or reduce the increase in $[\text{Ca}^{2+}]_i$. This can be done by resealing of the cellular membrane or reducing Ca^{2+} flux through Ca^{2+} channels in the sarcolemma and SR membrane.

Resealing

A lot of research has gone into developing agents that assist in resealing or stabilization of the membrane. Muscle damage can be considered as a continuous process of membrane degradation and repair. Assisting membrane repair may stop the vicious cycle, resulting in survival of the muscle fiber. The use of synthetic surfactants to stabilize membranes and thus restore cellular integrity has yielded promising results both *in vitro* and *in vivo*.⁷¹ (Membrane-sealing therapy will be reviewed elsewhere in this volume by Lee and Lee.)

Ca^{2+} Channel Blockers

A certain influx of Ca^{2+} occurs via the L-type Ca^{2+} channels in the outer membrane and in the T-tubules when the muscle is excited. If the muscle cell experiences an increased Ca^{2+} load, blocking any further influx may allow the muscle cell to maintain control over Ca^{2+} . Studies using the Ca^{2+} channel blockers have showed a reduction in the exercise-induced damage both in human and animal studies.^{72,73}

The ryanodine receptor Ca^{2+} -release channels in the SR likewise release Ca^{2+} into the cytoplasm following an action potential. Blocking of this (by, for example, dantrolene) has proven efficient during malignant hyperthermia and sepsis.⁷⁰

Inhibiting the Actions of Ca^{2+}

A second approach to prevent or reduce muscle damage is to prevent the consequences of the increased $[\text{Ca}^{2+}]_c$. This may be done by blocking the actions of calpain, PLA_2 , and ROS.

Calpain Inhibitors

Much discussion regarding the role of calpain in muscle damage has come from the conflicting evidence that an increase in $[\text{Ca}^{2+}]_c$ results in cellular damage, but in some cases this could not be prevented by the use of calpain inhibitors. This discrepancy may be the result of the different types of calpain, all with different characteristics. The calpain inhibitor calpastatin only inhibits calpains 1 and 2, with little effect on calpain 3. Leupeptin on the other hand inhibits all three calpains, but calpain 3 needs to undergo autolysis before leupeptin can bind to the active site.²² Ca^{2+} causes autolytic activation of single calpain 3 molecules, and these can then proteolytically activate other nearby calpain 3 molecules in a strongly self-reinforcing activation cascade.⁷⁴ Since leupeptin cannot bind before calpain 3 is autolyzed it may not be possible for leupeptin to prevent proteolytic activity of calpain 3 when $[\text{Ca}^{2+}]$ is appreciably above the threshold level for calpain activation.²⁵ However, if the activating $[\text{Ca}^{2+}]$ is relatively low (e.g., $\sim 2 \mu\text{M}$) the actions of calpain may be blocked by leupeptin.²⁵ This may be the case in DMD/*mdx*, where it was recently shown that muscle fiber degeneration in *mdx* mice was delayed by intramuscular administration of leupeptin.²⁹

PLA_2 Inhibitors

Inhibition of PLA_2 has been found to reduce enzyme release (e.g., CK and LDH) from skeletal muscle,^{31,32} although PLA_2 inhibitors failed to protect against myofibrillar damage.³³ Thus blocking PLA_2 activity may reduce further damage to the membrane, but does not provide protection for structural damage within the muscle fiber.

ROS Scavengers

There is little doubt that ROS are involved in the development of skeletal muscle damage over a range of situations. Production of ROS is dramatically accelerated during contractile activity, mainly owing to the increased oxygen consumption.³⁴ Many studies have shown that an increased Ca^{2+} load also leads to an increased production of ROS. In addition, as mentioned above (Section 3.3), the actions of ROS may result in an increased Ca^{2+} load on the muscle cell.

The effect of antioxidants in reducing muscle damage after exercise has been moderate, with some studies showing no effect and others showing small benefits. Recent studies have suggested that xanthine oxidase-mediated oxidative stress leads to useful cellular adaptations to exercise and that the practice of taking antioxidants prior to exercise may have to be re-evaluated.⁷⁵

Nevertheless antioxidant treatment may prove to be beneficial in other situations (e.g., sepsis), where a pathologic increase in ROS production leads muscle cell damage.

CONCLUDING REMARKS

There is good reason to believe that Ca^{2+} plays an important role in the development of many types of muscle damage. Situations of an increased influx of Ca^{2+} may initiate a vicious cycle, leading to muscle damage and death. Therefore, inhibiting the actions of Ca^{2+} may be important in preventing or reducing skeletal muscle damage. This may involve the use of a combination of compounds, as in combining membrane-stabilizing agents with antioxidants and calpain and PLA_2 inhibitors.

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