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Mitochondrial calcium regulation during and following contractions in skeletal muscle

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Abstract In skeletal muscle, resting cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{cyto}}$) homeostasis is exquisitely regulated by Ca^{2+} transport across the sarcolemmal, mitochondrial and sarcoplasmic reticulum (SR) membranes. Specifically, skeletal muscle contractile function is critically dependent on effective SR Ca^{2+} handling. However, more recent studies have revealed that mitochondria are essentially involved in $[\text{Ca}^{2+}]_{\text{cyto}}$ homeostasis during and following muscle contractions. We recently provided substantial support for the mitochondria as a major site for Ca^{2+} sequestration in skeletal muscle during recovery from fatiguing tetanic contractions under *in vivo* conditions. This review provides an overview of the role of skeletal muscle mitochondria in $[\text{Ca}^{2+}]_{\text{cyto}}$ regulation *in vivo* during and following muscle contractions.

Keywords : mitochondrial calcium homeostasis, skeletal muscle, contraction, mitochondrial calcium uniporter (MCU)

Introduction

Ca^{2+} is a highly versatile and precise intracellular controller that can regulate different cellular processes¹⁾. In skeletal muscle, cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{cyto}}$) homeostasis is exquisitely regulated by Ca^{2+} transport across the sarcolemma/plasma membrane (PM), mitochondria and sarcoplasmic reticulum (SR; Fig. 1). More recently, it was discovered that mitochondria play a major role in the dynamics of Ca^{2+} sequestration with evidence for increased mitochondrial Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{mito}}$) during/after tetanic contractions in skeletal muscle²⁻⁵⁾.

In the past few years we have succeeded in measuring $[\text{Ca}^{2+}]_{\text{cyto}}$ in rat skeletal muscle *in vivo* following repeated tetanic contractions⁶⁻⁹⁾. Using this novel approach, we investigated the mechanistic role for mitochondria in $[\text{Ca}^{2+}]_{\text{cyto}}$ regulation in skeletal muscle by examining a genetic mouse model of skeletal muscle specific peroxisome proliferator-activated receptor- γ coactivator-1 α overexpression (PGC-1 α OE). Interestingly, we observed improved $[\text{Ca}^{2+}]_{\text{cyto}}$ homeostasis during recovery from fatiguing tetanic contraction in PGC-1 α OE mice¹⁰⁾. This model displayed a selective upregulation of mitochondrial Ca^{2+} uptake-related proteins independent of SR function.

Based on these findings, we hypothesized that mito-

chondria play a crucial role in the buffering of $[\text{Ca}^{2+}]_{\text{cyto}}$ to maintain physiological Ca^{2+} regulation. This review considers the impact of the mitochondrial reticulum on $[\text{Ca}^{2+}]_{\text{cyto}}$ homeostasis during and following skeletal muscle contractions.

Structural components of Ca^{2+} transport across organelle membranes

SR membrane. The contraction-relaxation cycle of skeletal myocytes is regulated by transient increases of $[\text{Ca}^{2+}]_{\text{cyto}}$ above normal resting levels^{11,12)}. This process is initiated by sarcolemmal and transverse tubule depolarization, which triggers Ca^{2+} release from the SR via ryanodine receptors (RyRs)^{13,14)}. RyRs are a class of huge homotetrameric channels regulated by multiple factors including ions, small molecules, and proteins. Ca^{2+} release from RyRs is activated through charge-dependent conformational changes of the dihydropyridine receptor (DHPR)^{13,15)}. Calsequestrin (CSQ) is located in the SR lumen, and modulates the sensitivity of RyRs^{16,17)}. Dephosphorylated FKBP12 (composed of FK-506 binding proteins) is associated with a decreased probability and duration of RyR opening, which impairs SR Ca^{2+} release¹⁸⁾. SR Ca^{2+} -ATPase (SERCA) performs the critical function of promoting muscle relaxation by sequestering Ca^{2+} from the cytoplasm at the expense of ATP

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hydrolysis^{19,20}). In contrast, sarcolipin regulates Ca^{2+} reuptake by inhibiting the pumping activity of SERCA^{21,22}). Recently, muscle specific RNA (myoregulin), a transmembrane alpha helix expressed only in skeletal muscle, was identified as a functional micropeptide that regulates Ca^{2+} uptake into the SR during/following contractions²³).

Sarcolemmal membrane. During high Ca^{2+} transients, Ca^{2+} exchangers are supported by Ca^{2+} transporters, including Ca^{2+} channels and Ca^{2+} efflux pumps²⁴). The plasma membrane calcium ATPase (PMCA) and $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) are located in the PM/sarcolemma and promote Ca^{2+} efflux from the cytosol²⁵). In smooth muscle, PMCA isoform 4 is crucial for Ca^{2+} efflux activity of the PM²⁶), whereas PMCA isoform 1, located near junctional transverse tubules, is important in skeletal muscle²⁷). The NCX operates as a transmembrane electrochemical gradient coupled to the Na^+ influx and Ca^{2+} efflux system²⁸). In skeletal muscle, the NCX1 and NCX3 isoforms are highly expressed and are localized in the transverse tubules and sarcolemma^{25,29}). NCX1 is predominantly expressed

in slow oxidative fibers and NCX3 in fast glycolytic fibers³⁰). However, there is evidence that NCX may not serve to sequester Ca^{2+} , at least during tetanic contractions in skeletal muscle (single myofiber preparation in neonatal rats³⁰). Finally stretch-induced contractions lead to membrane damage allowing substantial “extra” Ca^{2+} entry through stretch-activated channels (SAC)^{31,32}).

Mitochondrial membrane. The mitochondria are vital for energy production via oxidative phosphorylation. In particular, regulation of mitochondrial Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{mito}}$) contributes to control of oxidative metabolism and ATP synthesis³³). In contrast, mitochondrial Ca^{2+} overload induces reactive oxygen species (ROS), sometimes leading to cell death⁴). As shown in Fig. 2, the mitochondrial reticulum is located adjacent to the SR and close to Ca^{2+} release units, providing structural evidence to support the hypothesis that the mitochondria and SR might function in a complimentary fashion to regulate $[\text{Ca}^{2+}]_{\text{cyto}}$ dynamics in skeletal muscle fibers³⁴). In particular, Ca^{2+} release from the SR through RyRs promotes Ca^{2+} uptake

Fig. 1 Schematic diagram of intracellular mechanisms for Ca^{2+} regulation and regulatory proteins in skeletal muscle. The contraction induces a Ca^{2+} release through the PM (sarcolemma) and by the SR and mitochondria. Subsequently, that Ca^{2+} is re-sequestered/removed from the cytosol by multiple exchangers and pumps. PM: Plasma membrane, SR: Sarcoplasmic reticulum, PMCA: Plasma membrane Ca^{2+} -ATPases, NCX: Na⁺/Ca²⁺ exchangers, SAC: Stretch-activated channels, NCX: Na⁺/Ca²⁺ exchangers, MCU: Mitochondrial calcium uniporter, RyRs: Ryanodine receptors, SERCA: Sarcoplasmic reticulum Ca^{2+} -ATPases. Ca^{2+} influx processes to the cytoplasm for contraction are shown in green color. Ca^{2+} uptake from the cytoplasm and relaxation is shown in orange color.

into the neighboring mitochondria^{35,36}. Mitofusin (Mfn) is a transmembrane GTPase that participates in fusion of the mitochondrial outer membranes of two adjacent mitochondria, which is regulated by Ca^{2+} uptake into the mitochondria³⁷. In cardiac muscle, Mfn2-mediated mitochondrial SR tethering facilitates Ca^{2+} cross-talk³⁸, and genetic ablation of Mfn2 was shown to create dysfunctional mitochondria-endoplasmic reticulum interactions and increase peak ATP-stimulated $[\text{Ca}^{2+}]_{\text{mito}}$ ³⁹. In fact, mitochondrial Ca^{2+} uptake is impaired in acute electroporated Mfn2 knockdown mice after tetanic contractions in skeletal muscle³. Skeletal muscle mitochondrial fusion based on Mfn1 is a key supporter of excitation contraction coupling⁴⁰. Thus, the increase in $[\text{Ca}^{2+}]_{\text{cyto}}$ buffering capacity may be supported by increased mitochondrial fusion as regulated by Mfn1 and Mfn2.

The inner mitochondrial membranes (IMM) contain several proteins that regulate mitochondrial Ca^{2+} uptake (Fig. 2). Specifically, the mitochondrial calcium uniporter (MCU), which binds Ca^{2+} with high affinity and promotes Ca^{2+} uptake in energized mitochondria, even at very low cytoplasmic concentrations, is believed to play a key role in mitochondrial Ca^{2+} handling^{41,42}. The MCU is regulated by mitochondrial Ca^{2+} uptake 1 (MICU1), MICU2 and essential MCU regulator (EMRE), which confers

Ca^{2+} sensitivity⁴³. MCU1 is a 40 kDa inner membrane protein that possesses two transmembrane spanning helices. MICUs are required for mitochondrial Ca^{2+} uptake through activity of MCU at high $[\text{Ca}^{2+}]_{\text{cyto}}$ ^{43,44}. EMRE is a 10 kDa protein with a transmembrane domain that spans the IMM and is involved in the interaction of MCU with MICU1 and MICU2. Because MCU requires the presence of EMRE, silencing EMRE prevents MICU1 and MICU2 co-immunoprecipitation with MCU⁴⁵.

Identification of MCU by Rizzuto and colleagues⁴² constituted perhaps the first evidence of a structural component to mitochondrial Ca^{2+} regulation. The importance of MCU function was demonstrated when mitochondria from MCU knockout mice were unable to rapidly uptake Ca^{2+} and displayed exercise intolerance that was related to changes in pyruvate dehydrogenase phosphorylation and activity⁴⁶. Silencing MCU inhibits mitochondrial Ca^{2+} uptake and accumulation in many cell types and physiological conditions^{41,47-50} reducing mitochondrial volume and increasing damage⁵². In contrast, overexpressing MCU increases mitochondrial volume and induces cristae proliferation⁵². Interestingly, MCU also regulates skeletal muscle hypertrophy and atrophy via PGC-1 α and insulin-like growth factor (IGF-1) as well as protein kinase B hypertrophic pathway regulation⁵¹.

Fig. 2 Schematic diagram of intracellular mechanisms for Ca^{2+} regulation by mitochondria in skeletal muscle. Note that the mitochondria also have an important sequestration role during the recovery process through a uniporter. Mfn: Mito fusion 1 and/or 2, MCU: Mitochondrial calcium uniporter, EMRE: Essential MCU regulator, MICU: Mitochondrial calcium uptake, ANT: Adenine nucleotide translocase, TT: T tubule. Electron micrograph shows adjoining SR and mitochondria by Eshima et al.¹⁰. Scale bar = 100 nm.

Role of mitochondria in Ca^{2+} regulation during skeletal muscle contraction

Both ATP and Ca^{2+} are required for skeletal muscle contractile function, and their availability is regulated by two major intracellular organelles, the SR and mitochondria. Accordingly, the rate of muscle contraction-relaxation cycling is widely believed to be dependent on the capacity for Ca^{2+} release and reuptake of the SR^{13,52,53}. In parallel with this SR function, $[\text{Ca}^{2+}]_{\text{mito}}$ increases during/after tetanic contractions in muscle *in vitro*, confirming that these organelles also play a role in regulating $[\text{Ca}^{2+}]_{\text{cyto}}$ ^{2,4,5,54,55}. Westerblad and colleagues showed that the increase in $[\text{Ca}^{2+}]_{\text{mito}}$ did not result in a significantly impaired force production in single muscle fibers⁵⁴. In addition, Tfam knockout⁴ and mitochondrial DNA mutator mouse⁵ mitochondrial myopathy models exhibit impaired fatigue resistance in the absence of altered $[\text{Ca}^{2+}]_{\text{cyto}}$ accumulation. However, mitochondrial Ca^{2+} uptake is a key supporter of excitation-contraction coupling in skeletal myotubes⁴⁰ and mitochondrial Ca^{2+} uptake is compromised in acutely

electroporated Mfn2 knockdown mice after tetanic contractions in the absence of changes in Ca^{2+} regulation proteins³.

Recently, in *in vivo* diabetic skeletal muscle, we demonstrated impaired Ca^{2+} handling (Fig. 3) and determined its independence from SR Ca^{2+} -ATPase protein content^{6,7}. Because diabetic muscle shows reduced mitochondrial enzyme activity⁵⁶, we proposed that mitochondrial, rather than SR, dysfunction might contribute to the impaired $[\text{Ca}^{2+}]_{\text{cyto}}$ regulation in these muscles. This hypothesis was supported by the finding that, in healthy mammalian muscle *in vivo*, physiologically stimulated $[\text{Ca}^{2+}]_{\text{cyto}}$ transients elevate $[\text{Ca}^{2+}]_{\text{mito}}$ levels⁵⁷. Subsequently, we demonstrated that increased mitochondrial content and function (PGC-1 α OE mice) in muscles were associated with accelerated restoration of baseline pre-contraction $[\text{Ca}^{2+}]_{\text{cyto}}$ following contractions¹⁰ (Fig. 4). This model showed significantly increased $[\text{Ca}^{2+}]_{\text{mito}}$ following contractions and pharmacological inhibition of SR. Furthermore, PGC-1 α OE induced a selective upregulation of mitochondria Ca^{2+} sequestration-related proteins, while their counterparts

Fig. 3 Diabetic fast-twitch (EDL, type II) skeletal muscles DIA are more fatigable than controls (CONT) and, after a prolonged fatiguing tetanic contraction, EDL $[\text{Ca}^{2+}]_{\text{cyto}}$ homeostasis cannot be recovered within a prolonged period. *Left*, typical example of changes in $[\text{Ca}^{2+}]_{\text{cyto}}$ following 120 sec (second) contraction in rat EDL muscle. Fluorescence images were captured continuously with no delay following contraction. Bar = 200 μm . *Right*, fluorescence was measured continuously from 120 to 600 s, and ratiometrically quantified data were graphed as changes from precondition (i.e., 120 s) levels (R_0). Values shown are expressed as means $\pm$ SE ($n = 6-8$). $P < 0.05$, significant difference between CONT and DIA (#) and significantly different from initial level for each condition (*). From Eshima et al.⁶.

in the SR were either unchanged or down-regulated. In particular, SR-mitochondrial Ca^{2+} cross-talk is thought to play an important role in the Ca^{2+} transients found in mammalian skeletal muscle, and is intrinsic to skeletal muscle $[\text{Ca}^{2+}]_{\text{cyto}}$ homeostasis⁵⁵. It is pertinent, therefore, that the RyRs were in far closer proximity to the mitochondrial outer membrane in PGC-1 α OE muscles¹⁰. Collectively, this evidence from *in vivo* skeletal muscle demonstrates a heretofore unappreciated role for skeletal muscle mitochondria in $[\text{Ca}^{2+}]_{\text{cyto}}$ regulation at rest and following fatiguing tetanic contractions.

Conclusion

The present review provides novel insights into the importance of mitochondria for $[\text{Ca}^{2+}]_{\text{cyto}}$ regulation in skeletal muscle. Our contention that the mitochondria constitute a crucial site for $[\text{Ca}^{2+}]_{\text{cyto}}$ regulation is supported by experimental models that reduce (i.e., diabetes) or augment (i.e., PGC-1 α OE) mitochondrial structure and function. Specifically, under *in vivo* conditions $[\text{Ca}^{2+}]_{\text{cyto}}$

homeostasis is grossly compromised in diabetic muscles. In marked contrast, PGC-1 α OE muscles, with their greater mitochondrial volume density, evince improved Ca^{2+} uptake via upregulation of mitochondrial Ca^{2+} uptake-related proteins that improve $[\text{Ca}^{2+}]_{\text{cyto}}$ homeostasis independent of SR function. Future research clarifying the dynamics of the relationship between mitochondrial Ca^{2+} uptake and $[\text{Ca}^{2+}]_{\text{cyto}}$ regulation *in vivo* during skeletal muscle contractions in healthy and disease models will be invaluable.

Conflict of Interests

No conflict of interests, financial or otherwise, are declared by the authors.

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Fig. 4 Improved $[\text{Ca}^{2+}]_{\text{cyto}}$ homeostasis during recovery from fatiguing tetanic contractions in PGC-1 α OE mice. *In vivo* cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{cyto}}$) profiles following 120-s contractions for tibialis anterior muscles from wild-type (WT) and peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α)-overexpressing (OE) mice. *Left*, Fura 2-AM fluorescence images were captured continuously with no delay following contractions. Scale bar = 200 μm . *Right*, fluorescence was measured before contraction and continuously for 450 s of recovery, and ratiometrically quantified data were graphed as changes from baseline levels (R_0). Values shown are expressed as means $\pm$ SE (WT, $n = 6$ mice; PGC-1 α OE, $n = 4$ mice). #Significant difference ($P < 0.05$) between WT and PGC-1 α OE. *Significantly different ($P < 0.05$) from initial level for each condition. From Eshima et al.¹⁰.

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