Mitochondrial Ca\textsuperscript{2+} uptake in skeletal muscle health and disease

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Muscle uses Ca\textsuperscript{2+} as a messenger to control contraction and relies on ATP to maintain the intracellular Ca\textsuperscript{2+} homeostasis. Mitochondria are the major sub-cellular organelle of ATP production. With a negative inner membrane potential, mitochondria take up Ca\textsuperscript{2+} from their surroundings, a process called mitochondrial Ca\textsuperscript{2+} uptake. Under physiological conditions, Ca\textsuperscript{2+} uptake into mitochondria promotes ATP production. Excessive uptake causes mitochondrial Ca\textsuperscript{2+} overload, which activates downstream adverse responses leading to cell dysfunction. Moreover, mitochondrial Ca\textsuperscript{2+} uptake could shape spatio-temporal patterns of intracellular Ca\textsuperscript{2+} signaling. Malfunction of mitochondrial Ca\textsuperscript{2+} uptake is implicated in muscle degeneration. Unlike non-excitable cells, mitochondria in muscle cells experience dramatic changes of intracellular Ca\textsuperscript{2+} levels. Besides the sudden elevation of Ca\textsuperscript{2+} level induced by action potentials, Ca\textsuperscript{2+} transients in muscle cells can be as short as a few milliseconds during a single twitch or as long as minutes during tetanic contraction, which raises the question whether mitochondrial Ca\textsuperscript{2+} uptake is fast and big enough to shape intracellular Ca\textsuperscript{2+} signaling during excitation-contraction coupling and creates technical challenges for quantification of the dynamic changes of Ca\textsuperscript{2+} inside mitochondria. This review focuses on characterization of mitochondrial Ca\textsuperscript{2+} uptake in skeletal muscle and its role in muscle physiology and diseases.

skeletal muscle, mitochondria, Ca\textsuperscript{2+}

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INTRODUCTION

ATP is the major currency of energy for sustaining life and is mostly produced in mitochondria. At the expense of other nutrient substrates and oxygen, mitochondria produce ATP that can be exchanged instantly whenever intracellular energy is required (Knowles, 1980). As described in the historical review by O’Rourke (O’Rourke, 2010), mitochondria, when initially discovered by Richard Altman in 1890, were called “bioplast”, meaning “life germs”. The word “mitochondria” was given by Carl Benda in 1898. For decades mitochondria were studied as the power house of cell, and soon it was realized that Ca\textsuperscript{2+} entry into mitochondria is required to stimulate the Krebs cycle and electron transport chain activity that result in enhanced ATP synthesis inside mitochondria (Balaban, 2002; Carafoli, 2014; Denton et al., 1980; Drago et al., 2011).

Ca\textsuperscript{2+} is fundamental to normal cellular function. Cells possess specialized mechanisms to ensure a tightly controlled intracellular Ca\textsuperscript{2+} level. These mechanisms involve complex interplay between intracellular Ca\textsuperscript{2+} storage, buffering and Ca\textsuperscript{2+} influx and efflux through the plasma membrane. The mitochondrial matrix has the ability to sequester Ca\textsuperscript{2+} when free cytosolic Ca\textsuperscript{2+} rises above a set point (Nicholls, 2005). Thus, mitochondria are recognized as one of the sub-cellular organelles participating in regulation of the intracellular Ca\textsuperscript{2+} homeostasis. Mitochondria are dynamic organelles that interact with the plasma membrane and the endoplasmic reticulum (ER) (Boncompagni et al., 2009; Eisner et al., 2013), and contribute to the recycling of Ca\textsuperscript{2+} back to the vicinal ER (Arnaudeau et al., 2001; Frieden et al., 2005). While intracellular Ca\textsuperscript{2+} signaling controls...
mitochondrial motility, distribution and function (Yi et al., 2004), reciprocally, mitochondria also modulates spatial and temporal intracellular Ca\(^{2+}\) levels.

Skeletal muscle contraction needs both Ca\(^{2+}\) and ATP. Thus, muscle physiology largely depends on two intracellular organelles: the sarcoplasmic reticulum (SR) for Ca\(^{2+}\) storage and release (Franzini-Armstrong and Jorgensen, 1994), and mitochondria for ATP synthesis (Russell et al., 2014). In non-muscle cells, the functional and physical coupling between ER and mitochondria is attributed to the inner-organellar tether proteins called mitofusin at the juxtaposition between the ER and mitochondria (de Brito and Scorrano, 2008). This type of structure was also found in skeletal muscle cells in which a tether like protein connects the SR and mitochondria (Boncompagni et al., 2009; Pietrangelo et al., 2015). These pivotal findings have heightened the role of mitochondria as a key player in the dynamic regulation of physiological Ca\(^{2+}\) signaling in skeletal muscle. Although it is believed that there is resemblance of mitochondrial structure and function among all cell types, the way by which mitochondrial Ca\(^{2+}\) uptake regulating intracellular Ca\(^{2+}\) signaling has specific features in skeletal muscle. Mitochondria in muscle cells face rapid changes of intracellular Ca\(^{2+}\) levels during contraction. Whether mitochondria Ca\(^{2+}\) uptake modifies Ca\(^{2+}\) signaling during excitation-contraction coupling has been a fundamental question in muscle physiology (O’Rourke and Blatter, 2009; Rossi et al., 2009). In order to answer this fundamental question, effort has been made to evaluate mitochondrial Ca\(^{2+}\) uptake in skeletal muscle under various physiological conditions. Characterization of mitochondrial Ca\(^{2+}\) uptake is a key step to understand the role of mitochondria in muscle physiology and diseases. This review focuses on characterization of mitochondrial Ca\(^{2+}\) uptake in skeletal muscle and its significance in skeletal muscle physiology and diseases.

MITOCHONDRIAL Ca\(^{2+}\) UPTAKE REGULATES ENERGY PRODUCTION IN SKELETAL MUSCLE

Ca\(^{2+}\) is a critical messenger not only for muscle contraction, but also for promoting mitochondrial ATP production. In mammalian cells, Ca\(^{2+}\) is a key regulator of ATP production (Griffiths and Rutter, 2009). Four important mitochondrial dehydrogenase involved in the direct supply of NADH (reduced nicotinamide adenine dinucleotide) and FADH (reduced flavin adenine dinucleotide) for ATP production were found to be regulated by Ca\(^{2+}\) inside mitochondria (Denton, 2009). A transient increase of free Ca\(^{2+}\) concentration is required to stimulate electron transport chain (ETC) of mitochondria in cardiac cells (Gueguen et al., 2005; Territo et al., 2000). The role of mitochondrial Ca\(^{2+}\) uptake in cardiac muscle energy metabolism has been widely studied (Balaban, 2002; Brookes et al., 2004).

In skeletal muscle, ATP demand increases ~100 times during rapid muscle contraction. Such high demand of ATP cannot be fulfilled by the finite amount ATP normally stored inside the skeletal muscle. Muscle contraction requires fast and sustained ATP production, which is fulfilled primarily by mitochondria (Porter and Wall, 2012). As such, skeletal muscle is known to be a tissue of high energy demand with mitochondria occupying 10%–15% of the fiber volume and densely packed within muscle cells (Eisenberg, 1983). In skeletal muscle, mitochondria are located largely within the I-bands, surrounding the SR network (Eisenberg, 1983). Importantly, mitochondria are found to be linked to the SR in skeletal muscle by developmentally regulated tethering structures (Boncompagni et al., 2009; Pietrangelo et al., 2015). This intimate juxtaposition of the SR and mitochondria, together with the ability of mitochondria to take up Ca\(^{2+}\) from their surroundings, allows the movement of Ca\(^{2+}\) between these organelar systems (Bianchi et al., 2004; Csordas and Hajnoczky, 2009; Rizzuto and Pozzan, 2006; Santo-Domingo and Damaurex, 2010). These movements are believed to help tailor mitochondrial metabolism and ATP synthesis to the demand of muscle contraction. Early studies of intact skeletal muscle observed an increase in NADH/NAD\(^{+}\) during the transition from resting to working status, suggesting that an enhanced intracellular Ca\(^{2+}\) level promotes mitochondrial metabolism in skeletal muscle (Duboc et al., 1988; Kunz, 2001; Sahlin, 1985). Later, using isolated mitochondria derived from skeletal muscle, Kavanagh et al. confirmed that an elevation in mitochondrial Ca\(^{2+}\) was able to stimulate oxidative phosphorylation (Kavanagh et al., 2000). As discussed in the review article by Rossi et al., mitochondrial Ca\(^{2+}\) uptake should assist with stimulation of aerobic ATP production in order to balance increased ATP consumption associated with cross bridge cycling and SERCA-mediated Ca\(^{2+}\) sequestration during muscle contraction (Rossi et al., 2009).

EVALUATION OF MITOCHONDRIAL Ca\(^{2+}\) UPTAKE IN SKELETAL MUSCLE

In order to understand the role of mitochondrial Ca\(^{2+}\) uptake in skeletal muscle physiology, it is vital to evaluate the amount and the kinetics of mitochondrial Ca\(^{2+}\) uptake in skeletal muscle cells under physiological conditions. The early studies on mitochondrial Ca\(^{2+}\) uptake were performed on isolated mitochondria (Deluca and Engstrom, 1961; Mraz, 1962). These studies showed that isolated mitochondria from rat kidney were able to take up 60% of Ca\(^{2+}\) from the surrounding medium (Deluca and Engstrom, 1961). The kinetics of mitochondrial Ca\(^{2+}\) uptake was well documented in the isolated mitochondria from the liver and heart (Carafoli and Crompton, 1978; McMillin-Wood et al., 1980). Sembrowich et al. was the first to explore the Ca\(^{2+}\) uptake by mitochondria derived from different types of skeletal muscle both from rats and rabbits (Sembrowich et al., 1985). Using direct patch-clamp recording on the inner mi-
tochondrial membrane, Fieni et al. recorded the mitochondrial Ca\textsuperscript{2+} uptake activity in mitoplasts isolated from mitochondria of different types of including skeletal muscle (Fieni et al., 2012). These \textit{in vitro} studies also suggested a potential influence of mitochondrial Ca\textsuperscript{2+} uptake on cytosolic Ca\textsuperscript{2+} signaling during muscle contraction. However, such conclusion needs validation from \textit{in vivo} studies. Specifically, it requires characterization of mitochondrial Ca\textsuperscript{2+} uptake in intact muscle cells under physiological conditions.

There are a few probes available to monitor Ca\textsuperscript{2+} fluxes into and out of mitochondria in live cells. The commercially available fluorescent dye Rhod-2 has been widely used in investigating mitochondrial Ca\textsuperscript{2+} handling in cultured cells because the acetoxymethyl (AM) ester of rhod-2 (Rhod-2-AM) preferentially targets mitochondria (see review (Pozzan and Rudolf, 2009)). Rhod-2 has been used to measure mitochondrial Ca\textsuperscript{2+} uptake in cultured skeletal muscle myotubes under electric stimulation (Eisner et al., 2010). The shortcoming is that Rhod-2 is not a ratiometric dye (Fonteriz et al., 2010). The uneven distributions of the dye among individual mitochondria can also cause problems for quantification of mitochondrial Ca\textsuperscript{2+} concentration changes based on fluorescence intensity (Lakin-Thomas and Brand, 1987). Rhod-2 has also been used to monitor mitochondrial Ca\textsuperscript{2+} uptake in intact skeletal muscle fibers following repeated tetanic stimulation (Ainbinder et al., 2015; Bruton et al., 2003). However, the specific targeting of Rhod-2-AM to mitochondria in intact muscle fibers was challenging. To avoid the Rhod-2 signals from outside mitochondria, Shkryl and Shirokova recorded mitochondrial Ca\textsuperscript{2+} uptake during caffeine-induced Ca\textsuperscript{2+} release in permeabilized rat skeletal muscle fibers (Shkryl and Shirokova, 2006). In this case, cell membrane permeabilization of the muscle fibers allowed the non-targeted Rhod-2 dye to leak out of the cytosol. However, since muscle fibers with permeabilized membrane no longer respond to physiological stimulations (i.e. membrane depolarization), the condition employed in such a study is not suitable for quantitative and specific evaluation of mitochondrial Ca\textsuperscript{2+} uptake in intact skeletal muscle cells under physiological conditions.

Due to various limitations, quantitative measurement of mitochondrial Ca\textsuperscript{2+} uptake in skeletal muscle remains to be challenging. GFP and other functionally similar fluorescent proteins have modernized the research in cell biology (Tsien, 1998). Owing to mutations and variations in gene sequences, genetically encoded fluorescent proteins have been developed as Ca\textsuperscript{2+} biosensors with varying properties including differences in fluorescence spectra, Ca\textsuperscript{2+} binding affinities and kinetics as well as those that change spectral properties upon binding to calcium (Palmer et al., 2006). The rapid growth of molecular biology techniques also allows the genetically encoded Ca\textsuperscript{2+} biosensors to target to specific sub-cellular organelles such as mitochondria (Pozzan and Rudolf, 2009). Thus, organelle-targeted ratiometric Ca\textsuperscript{2+} biosensors has become a better choice for characterization of mitochondrial Ca\textsuperscript{2+} uptake in skeletal muscle under physiological conditions. Using a mitochondrial targeted biosensor (2mtYC2), Rudolf et al. demonstrated that a single twitch could cause measurable dynamic changes in mitochondrial Ca\textsuperscript{2+} levels in live skeletal muscle fibers. However, they also noted some limitations of 2mtYC2 for mitochondrial Ca\textsuperscript{2+} measurement in muscle cells, for instance, YC2 had a small dynamic range with an increase of the emission ratio <26% in the cytosol and <14% in mitochondria during muscle contraction (Rudolf et al., 2004). Subsequently, Palmer et al. developed a new version of mitochondrial targeted Ca\textsuperscript{2+} biosensor, 4mtD3cpv, which has a dynamic ratio range of 5.1 (Palmer et al., 2006). Upon testing 4mtD3cpv on live skeletal muscle fibers under voltage-clamp conditions, Zhou et al. found that while 4mtD3cpv showed a significant improvement in monitoring mitochondrial Ca\textsuperscript{2+} levels in live muscle fibers with an increased dynamic ratio range, the kinetics of the detected signal set some limitations for quantitatively calculating the changes of the mitochondrial Ca\textsuperscript{2+} level (Zhou et al., 2008). As an alternative, YC3.6, another Ca\textsuperscript{2+} biosensor constructed by Nagai and colleagues (Nagai et al., 2004), with a dynamic ratio range of 5.6 and apparent $K_d$ of 0.25 μmol L\textsuperscript{-1} was later tested by Yi et al. in live skeletal muscle fibers (Yi et al., 2011). By introducing a mitochondrial targeting sequence (Wang et al., 2008) at the 5′-end of YC3.6 cDNA, they developed a mitochondrial targeting Ca\textsuperscript{2+} biosensor, mt11-YC3.6. The highly specific mitochondrial expression of mt11-YC3.6 and the simple kinetics of the recorded YC3.6 ratio signal allowed quantitative evaluation of the dynamic changes of free Ca\textsuperscript{2+} levels inside mitochondrial matrix in skeletal muscle fibers in response to a Ca\textsuperscript{2+} release transient induced by cell membrane depolarization under whole-cell voltage clamped conditions. This study shows that at the peak of the voltage-induced Ca\textsuperscript{2+} release, the mitochondrial Ca\textsuperscript{2+} uptake contributes to around 10%–18% of the total Ca\textsuperscript{2+} removal, and the average mitochondrial Ca\textsuperscript{2+} influx is around 4.1±1.0 μmol L\textsuperscript{-1} ms\textsuperscript{-1} (Yi et al., 2011). This study represents the first quantitative characterization of mitochondrial Ca\textsuperscript{2+} uptake and its role in shaping the cytosolic Ca\textsuperscript{2+} signaling in skeletal muscle during excitation-contraction coupling.

**IMPAIRED SKELETAL MUSCLE MITOCHONDRIAL Ca\textsuperscript{2+} SIGNALING IN MUSCLE DISEASES**

Mitochondrial Ca\textsuperscript{2+} uptake plays vital roles in life and death of the cell. Impaired mitochondrial Ca\textsuperscript{2+} uptake is observed in various skeletal muscle myopathies and neuromuscular diseases. Defective intracellular Ca\textsuperscript{2+} signaling is associated with degeneration of skeletal muscle cells in aging (Dellino, 2002; Weisleder et al., 2006) and muscular dystrophy (mdx) (De Backer et al., 2002; DiFranco et al., 2008; Han...
Ca2+ are mainly controlled by the SR, which forms a network that is intimately associated with mitochondria. This close spatial proximity between the SR and mitochondria, together with the ability of mitochondria to take up Ca2+, suggests that mitochondria could play an important role in shaping intracellular Ca2+ signaling in muscle cells. However, whether mitochondrial Ca2+ uptake is large and rapid enough to modulate physiological Ca2+ transients in skeletal muscle and whether alterations in mitochondrial Ca2+-buffering capacity contribute to muscle dysfunction under pathophysiological conditions are fundamental questions for understanding muscle degeneration in various diseases. A direct evidence of mitochondrial regulation on the SR Ca2+ release activity in live skeletal muscle cells was obtained from the study on an amyotrophic lateral sclerosis (ALS) mouse model (G93A) with transgenic overexpression of the human ALS-associated SOD1(G93A) mutant (Zhou et al., 2010). The G93A muscle fibers display localized depolarization of mitochondrial inner membrane potential in the fiber segment near the neuromuscular junction. The depolarized mitochondria lose the driving force for Ca2+ uptake, which impairs mitochondrial Ca2+ buffering capacity. The fiber segments with depolarized mitochondria shows greater osmotic stress-induced Ca2+ release activity, which can include propagating Ca2+ waves. Those Ca2+ waves are confined to regions of depolarized mitochondria and stop propagating shortly upon entering the regions of normal, polarized mitochondria. Uncoupling of mitochondrial membrane potential with FCCP or inhibition of mitochondrial Ca2+ uptake by Ru360 also led to cell-wide propagation of such Ca2+ release events. These data reveals that mitochondrial Ca2+ uptake is large and rapid enough to shape cytosolic Ca2+ signaling in skeletal muscle under physiological conditions.

The ALS muscle fibers provide a unique opportunity to characterize the mitochondrial Ca2+ uptake under physiological conditions. The localized mitochondrial defect in the ALS muscle fibers allows for examination of mitochondrial contribution to Ca2+ removal during excitation-contraction coupling by comparing Ca2+ transients in regions with normal and depolarized mitochondria in the same muscle fiber. Using whole cell voltage-clamp technique, Yi et al. showed that Ca2+ transients elicited by membrane depolarization in the fiber segment with depolarized mitochondria displayed increased amplitude of ~10%. Using the mitochondria-targeted Ca2+ biosensor (mt11-YC3.6) expressed in ALS muscle fibers, these authors recorded the dynamic change of mitochondrial free Ca2+ levels during voltage-induced SR Ca2+ release and detected a reduced Ca2+ uptake by mitochondria in the fiber segment with depolarized mitochondria, which mirrored the elevated Ca2+ transients in the cytosol in the same region (Yi et al., 2011). This study provides a direct demonstration of the importance of mitochondrial Ca2+ uptake in shaping cytosolic Ca2+ signaling in skeletal muscle during excitation-
contraction coupling and suggests that the reduced Ca\(^{2+}\) buffering capacity of mitochondria likely contributes to muscle degeneration in ALS.

Although, it was well known that mitochondria from all cell types were able to take up Ca\(^{2+}\) and that the channel or transport responsible for mitochondrial Ca\(^{2+}\) uptake was defined as mitochondrial Ca\(^{2+}\) uniporter (MCU), the molecular identity of the putative MCU had remained mysterious for decades (Carafoli, 2014; Drago et al., 2011; Starkov, 2010). It was not until 2011 when two research groups independently identified the gene that encodes MCU, a transmembrane protein located to the inner mitochondrial membrane (Baughman et al., 2011; De Stefani et al., 2011). This new progress has further advanced the investigation of the role of mitochondrial Ca\(^{2+}\) uptake in skeletal muscle health and diseases. Pan et al. generated a global knockout mouse model (MCU-/-). The MCU-/- mice survived well with a smaller body size, but showed impaired skeletal muscle performance along with absence of mitochondrial Ca\(^{2+}\) uptake in isolated skeletal muscle mitochondria, indicating that mitochondrial Ca\(^{2+}\) uptake plays an important role in skeletal muscle development and performance (Pan et al., 2013). Recently, direct evidence of MCU-dependent mitochondrial Ca\(^{2+}\) uptake in protecting denervation-induced skeletal muscle atrophy was provided by Mammucari et al. and Chemello et al., in which, the authors have shown that virus-mediated overexpression or silencing of MCU had significant impact on skeletal muscle atrophy shown that virus-mediated overexpression or silencing of MCU had significant impact on skeletal muscle atrophy. This new progress has further advanced the investigation of the role of mitochondrial Ca\(^{2+}\) uptake in skeletal muscle function. While the Ca\(^{2+}\) influx into mitochondria is required for promoting ATP synthesis, excessive Ca\(^{2+}\) accumulation in mitochondria initiates a series of molecular malfunctions leading to mitochondrial damage and cell death. Under diseased conditions, such as muscular dystrophy, gene-mutation related myopathies and aging, enhanced SR Ca\(^{2+}\) release activity overloads mitochondria with Ca\(^{2+}\), leading to mitochondrial dysfunction and muscle cell degeneration. In those cases, mitochondrial damage seems to be a consequence of extensive elevation of cytosolic Ca\(^{2+}\) levels. In ALS G93A skeletal muscle, the mitochondrial membrane potential is depolarized, which leads to a reduced Ca\(^{2+}\) buffering capacity of mitochondria. This reduced mitochondrial Ca\(^{2+}\) uptake further overloads those polarized mitochondria with Ca\(^{2+}\) and causes further mitochondrial damage in the same cell. In this case, the compromised mitochondrial Ca\(^{2+}\) uptake is a leading cause of the disrupted intracellular Ca\(^{2+}\) signaling that initiates muscle cell degeneration. In summary, any dysregulation in the amount and kinetics of mitochondrial Ca\(^{2+}\) uptake will cause mitochondrial dysfunction and abnormal intracellular Ca\(^{2+}\) signaling that leads to muscle cell degeneration. It is predicted that identification of molecular basis associated with mitochondrial Ca\(^{2+}\) uptake will further advance the understanding of the role of mitochondrial Ca\(^{2+}\) uptake in skeletal muscle health and diseases.

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