Effects of nutritional status on mitochondrial Ca$^{2+}$ handling
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Efeitos do estado nutricional no manejo de Ca$^{2+}$ mitocondrial

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Mitochondria are central players in cell metabolism, responsible for the vast majority of ATP production in most cells. Although originally thought to be passive organelles focused only in keeping cellular ATP at adequate levels, complex interplay between mitochondrial function and cell signaling has been largely recognized over the last decades. Not surprisingly, given their role, changes in nutritional status promoted by chronic interventions like caloric restriction or short-term situations like fasting in animals or nutrient deprivation in cultured cells are one of the main factors that can activate those signaling mechanisms. One particular way in which this mitochondria-cell crosstalk can occur is through mitochondrial Ca$^{2+}$ handling, a process in which Ca$^{2+}$ signals generated by the cell are able to translate into elevations in mitochondrial matrix [Ca$^{2+}$] due to the presence of the mitochondrial Ca$^{2+}$ uniporter in the organelle. While the impact of mitochondrial Ca$^{2+}$ handling on cellular function has been widely studied, the conditions which can modulate the process of mitochondrial Ca$^{2+}$ handling itself are still not well characterized. In this work, we sought to test the effects of different interventions linked to nutritional status on mitochondrial Ca$^{2+}$ handling. We found that caloric restriction, physiological fasting and modulations of mitochondrial dynamics resulted in modulation of mitochondrial Ca$^{2+}$ handling through changes in their maximal
Ca\(^{2+}\) retention capacity or Ca\(^{2+}\) uptake rates. These changes were, measured by following mitochondrial Ca\(^{2+}\) uptake using different strategies, employing the fluorescent Ca\(^{2+}\) probe Ca\(^{2+}\) Green 5N for experiments in isolated mitochondria and permeabilized cells and the cytosolic probe Fura2-AM in intact cells. Caloric restriction resulted in higher calcium uptake and retention in liver mitochondria, protecting against pathological conditions of Ca\(^{2+}\) overload during ischemia/reperfusion. On the other hand, overnight and short term fasting resulted in lower mitochondrial Ca\(^{2+}\) retention and oxidative phosphorylation capacity in the liver. Modulating mitochondrial morpholoy in C2C12 myoblasts showed that more fragmented mitochondria were less capable of taking up Ca\(^{2+}\), while more fusioned mitochondria showed the opposite phenotype. This modulation in Ca\(^{2+}\) handling through changes in mitochondrial morphology interfered with the process of Store-Operated Ca\(^{2+}\) entry in the cells, showing that these modulations can have impacts in physiological contexts as well. Overall, this work both establishes novel mechanisms of modulation of mitochondrial Ca\(^{2+}\) handling and demonstrates their relevance both in pathology and normal cellular physiology.

**Keywords:** Mitochondria, Calcium, Liver, Cell death, Signaling, Myoblasts
RESUMO

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Mitocôndrias possuem um papel central no metabolismo das células, sendo responsáveis pela maioria da produção de ATP na maioria dos tipos celulares. Embora originalmente se pensasse nas mitocôndrias como organelas estáticas, focadas somente em manter os níveis adequados de ATP na célula, a interação entre a função mitocondrial e a sinalização celular tem sido fortemente reconhecida nas últimas décadas. Dado este papel, não é surpreendente que mudanças no estado nutricional, tanto crônicas como na restrição calórica quanto em situações como o jejum em animais e a privação de nutrientes em cultura de células foram demonstradas como sendo um dos principais fatores que podem ativar estes mecanismos de sinalização. Uma das formas em que esta interação entre a mitocôndria e a célula ocorre é através do manejo de Ca^{2+} mitocondrial, um processo em que sinais de Ca^{2+} gerados pela célula podem resultar em aumentos na [Ca^{2+}] na matriz mitocondrial devido à presença do uniportador de Ca^{2+} mitocondrial na organela. Embora o impacto do manejo de Ca^{2+} mitocondrial na função da célula tenha sido amplamente estudado, a regulação do processo de manejo de Ca^{2+} mitocondrial em si não é bem conhecida. Neste trabalho, nós nos propusemos a testar os efeitos de diferentes intervenções ligadas ao estado nutricional no manejo de Ca^{2+} mitocondrial e o possível impacto destas modulações na.
capacidade de retenção e na taxa de captação de Ca\textsuperscript{2+} mitochondrial. As intervenções estudadas foram a restrição calórica, jejum e mudanças na dinâmica mitochondrial, e todas elas resultando em mudanças no manejo de Ca\textsuperscript{2+} mitochondrial, que foram medidos acompanhando a captação de Ca\textsuperscript{2+} em mitocôndrias isoladas ou células permeabilizadas utilizando a sonda Ca\textsuperscript{2+} Green 5N e em células intactas utilizando a sonda de Ca\textsuperscript{2+} citosólica Fura2-AM. Enquanto a restrição calórica resultou em uma maior capacidade de retenção de Ca\textsuperscript{2+} e em maiores taxas de captação, protegendo contra as condições patológicas de desregulação de Ca\textsuperscript{2+} observadas durante a isquemia/reperfusão, o jejum curto ou pela duração da noite resultou em uma diminuição na capacidade de retenção de Ca\textsuperscript{2+} e na oxidação fosforilativa mitochondrial. As mudanças observadas modulando a dinâmica mitocôndria (feitas utilizando-se mioblastos da linhagem C2C12) revelaram que mitocôndrias mais fragmentadas são menos capazes de captar Ca\textsuperscript{2+}, enquanto mitocôndrias mais fusionadas possuem o fenótipo oposto. Essas mudanças no manejo de Ca\textsuperscript{2+} mitochondrial interferem com o processo de “Store-Operated Ca\textsuperscript{2+} entry” nestas células, demonstrando que essas modulações da captação de Ca\textsuperscript{2+} mitochondrial também podem ser relevantes em contextos fisiológicos. Em resumo, este trabalho ajudou a estabelecer novos mecanismos de modulação do manejo de Ca\textsuperscript{2+} mitochondrial que podem ser relevantes tanto em condições patológicas quanto na fisiologia normal das células.

**Palavras chave:** Mitocôndria, Cálcio, Fígado, Morte Celular, Sinalização, Mioblastos
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CHAPTER I – Introduction and goals
1. Introduction

1.1 Mitochondrial calcium handling and its role in energy metabolism and calcium signalling

Mitochondria are well known for their role in sustaining ATP production to supply the immense variety of cellular processes which require it, allowing for a much higher yield of ATP per molecule of glucose through oxidative phosphorylation than the two ATP molecules obtained as the net result of glycolysis alone. Due to its essential role in energy maintenance, extensive loss of mitochondrial oxidative phosphorylation capacity commonly leads to necrotic cell death secondary to the resulting ATP-depletion and subsequent failure to maintain basic processes essential for overall cellular homeostasis (reviewed in Nieminien, 2003). Mitochondria are also well known for being involved in cell death through other mechanisms, be it indirectly as in the excessive production of oxidants by the electron transport chain during certain stress conditions (Kowaltowski, Castilho and Vercesi, 2001) or directly through the release of pro-apoptotic factors like cytochrome c to the cytosol during apoptosis (Liu et al, 1996). Rather than being static organelles focused only on supplying ATP to the cell, mitochondria have been consistently shown to be highly dynamic, not only having their structure and function regulated by the cell within different physiological contexts, but also participating in several signaling pathways which allow them to interfere with cell function (reviewed in Tait and Green, 2012). Examples of this dynamic interplay include the induction of mitochondrial fusion in starved cells as a protective mechanism against energetic failure (Rambold et al, 2011), modulation of enzymatic activities by promotion of protein thiol oxidation through mitochondrially derived oxidants (reviewed in, Finkel, 2012), regulation
of stem cell differentiation by mitochondrial dynamics (Forni et al, 2016) and overall regulation of immune T cell activation and function (reviewed in Liu and Ho, 2018). Many new roles are being discovered, further revealing the importance of mitochondria in signaling and regulation of basic cell physiology processes.

One particular way in which mitochondria can both impact and be impacted by cell physiology occurs through mitochondrial Ca$^{2+}$ handling. Mammalian mitochondria have long been known to electrogenically uptake high levels of Ca$^{2+}$ into the mitochondrial matrix, driven by the negative-inside membrane potential. This allows Ca$^{2+}$ uptake even when [Ca$^{2+}$] in the mitochondrial matrix is several orders of magnitude higher than external [Ca$^{2+}$] (Santos-Domingo and Demareux, 2010).

Mitochondrial Ca$^{2+}$ uptake is attributed to the presence of a mitochondrial Ca$^{2+}$ channel known as the mitochondrial calcium uniporter (MCU) and its regulatory proteins MICU1, MICU2 and EMRE, usually referred collectively as the MCU complex (Santos-Domingo and Demareux, 2010; Sancak et al, 2013). Although the relevance of mitochondrial Ca$^{2+}$ uptake in cell physiology was at first questioned by some groups due to the high (and seemingly non-physiological) [Ca$^{2+}$] threshold needed to observe mitochondrial Ca$^{2+}$ uptake in isolated mitochondria, it is well known today that mitochondrial Ca$^{2+}$ uptake occurs under many physiological conditions and is relevant in normal cell function (reviewed in Patron et al, 2013; Mammucari et al, 2018). In most cases this uptake is made possible by the position of populations of mitochondria near Ca$^{2+}$ release/entry sites. Upon activation of Ca$^{2+}$ release from these storage sites, local high [Ca$^{2+}$] microdomains form, allowing for the concentration threshold for mitochondrial Ca$^{2+}$ uptake to be reached (reviewed in Rizzuto et al, 2009). Mitochondria with lower [Ca$^{2+}$] activation thresholds for Ca$^{2+}$ uptake have also been described, seemingly
because of different MICU1:MCU protein stoichiometries in the uniporter complex (Paillard et al, 2017), which would make Ca\textsuperscript{2+} uptake possible even outside of these high Ca\textsuperscript{2+} microdomain contexts.

While mitochondrial Ca\textsuperscript{2+} uptake occurs essentially only through the MCU complex [candidates for secondary mechanisms like mitochondrial ryanodine receptors and UCP2 have been proposed, but their role in mitochondrial Ca\textsuperscript{2+} uptake is highly controversial (reviewed in Rizzuto et al, 2009, Pan et al, 2013)], the process of mitochondrial Ca\textsuperscript{2+} extrusion occurs separately. Two well-defined processes for Ca\textsuperscript{2+} extrusion, both which have been known to occur for decades through functional analysis, have only recently been associated to specific proteins (reviewed in Mammucari et al, 2018). One of these processes occurs through the exchange of Ca\textsuperscript{2+} ions for Na\textsuperscript{+} ions, widely accepted to be mediated by the NCLX protein (Palty et al, 2009; reviewed in Boyman et al, 2013), although there is still some debate on whether its exchange stoichiometry is electrogenic or neutral (reviewed in Boyman et al, 2013). The other Ca\textsuperscript{2+} extrusion process occurs through the exchange of Ca\textsuperscript{2+} ions for H\textsuperscript{+} ions (reviewed in Mammucari et al, 2018). The LETM1 protein is suggested to be the mediator of the later (Jiang, Zhao and Clapham, 2009), but the acceptance of its role is not as uncontroversial as the role of NCLX in mitochondrial Ca\textsuperscript{2+}/Na\textsuperscript{+} exchange (de Marchi et al, 2014).

Ca\textsuperscript{2+} is a ubiquitous second messenger, normally maintained at very low concentrations in the cytoplasm under basal conditions (~100 nM), while [Ca\textsuperscript{2+}] is usually around 1-2 mM in the extracellular medium and within the range of some hundreds of micromolar inside the intracellular Ca\textsuperscript{2+} stores such as the endoplasmic reticulum (ER) or sarcoplasmic reticulum (SR) (reviewed in Bagur and Hajnóczky, 2017;
such a far from equilibrium difference in Ca\(^{2+}\) concentrations between the cytoplasm and those nearby environments is mainly attributed to the active transport of the ion from the cytoplasm, both to the extracellular milieu by the plasma membrane Ca\(^{2+}\)-ATPase (PMCA) and to the ER/SR through the SERCA pump (which is also a Ca\(^{2+}\)-ATPase). The maintenance of such a difference in concentration allows for the generation of controlled transient cytoplasmic [Ca\(^{2+}\)] elevations through mechanisms involving the opening of Ca\(^{2+}\) channels and the subsequent mobilization of Ca\(^{2+}\) ions from these high concentration environments to the lower concentration environment of the cytoplasm, resulting in temporary raises in cytoplasmic [Ca\(^{2+}\)] (usually not surpassing 1 µM in physiological conditions). These are later normalized by the aforementioned active transport mechanisms of the ion to the outside the cell and into the ER/SR (reviewed in Bagur and Hajnóczky, 2017; Clapham, 1995). Temporary raises caused by Ca\(^{2+}\) mobilization are able to modulate protein function and enzymatic activities through Ca\(^{2+}\) binding to their Ca\(^{2+}\) binding sites, thus eliciting physiological responses and making Ca\(^{2+}\) signals a highly versatile signaling tool (reviewed in Clapham, 1995). The most common manifestations of Ca\(^{2+}\) signaling consist in either a transient overall sustained elevation in cytosolic [Ca\(^{2+}\)] or an oscillatory pattern of several cycles of Ca\(^{2+}\) elevation followed by normalization, known as Ca\(^{2+}\) waves. While the first is mostly dependent on the extent and duration of [Ca\(^{2+}\)] elevation, the later has been shown to be more dependent on the frequency of [Ca\(^{2+}\)] peaks of the waves, with different frequencies being differentially decoded by the Ca\(^{2+}\) signaling machinery and thus being able to elicit different responses (reviewed in Smedler and Uhlén, 2014). Interestingly enough, different Ca\(^{2+}\) signals with different patterns have been shown to elicit very distinct responses, even within the same cell (reviewed in Smedler and Uhlén,
2014), a common signaling mechanism deployed in a wide diversity of processes (reviewed in Clapham, 1995; Smedler and Uhlén, 2014). While cytosolic \([\text{Ca}^{2+}]\) elevation during physiological \(\text{Ca}^{2+}\) signaling is a regulated process and usually does not surpass an overall micromolar concentration throughout the cytoplasm, uncontrolled elevation of cytoplasmic \([\text{Ca}^{2+}]\), a condition usually referred to as cellular \(\text{Ca}^{2+}\) overload, can reach much higher concentrations. High non-physiological concentrations of \(\text{Ca}^{2+}\) in the cytoplasm lead to cellular dysfunction, which can ultimately result in cell death (reviewed in Clapham, 1995). It is worth noticing, however, that while cellular \(\text{Ca}^{2+}\) overload can occur due to unregulated overactivation of \(\text{Ca}^{2+}\) channels responsible for physiological \(\text{Ca}^{2+}\) signaling [i.e. by the overactivation of NMDA receptors during excitotoxicity (reviewed in Dong, Wang and Qin, 2009)], it can also result from different conditions, such as the impairment of cytosolic \(\text{Ca}^{2+}\) extrusion and ER \(\text{Ca}^{2+}\) uptake mechanisms due to bioenergetic failure observed in ischemia/reperfusion (reviewed in Kalogeris et al, 2012).

Mitochondrial \(\text{Ca}^{2+}\) handling has been shown to have an impact both under physiological \(\text{Ca}^{2+}\) signaling conditions (reviewed in Mammucari et al, 2018; Rizzuto et al, 2012) and pathological cellular \(\text{Ca}^{2+}\) overload (reviewed in Nieminen, 2003; Orrenius, Zhivotovsky and Nicotera, 2003). Under physiological contexts, mitochondrial \(\text{Ca}^{2+}\) uptake has been shown to regulate \(\text{Ca}^{2+}\) release activity of IP3 receptors (IP3R, Hajnóczky, Hager and Thomas, 1999) and ryanodine receptors (RyR, Jackson and Thayer, 2006), both of which act as regulated \(\text{Ca}^{2+}\) channels present in the ER which are able to initiate cytosolic \(\text{Ca}^{2+}\) signals upon activation (reviewed in Clapham, 1995). Such regulation occurs due to the fact that both of these receptors are positively modulated by external \(\text{Ca}^{2+}\), which allows for a process of positive feedback exerted by
the high \([\text{Ca}^{2+}]\) microenvironment created after initial \(\text{Ca}^{2+}\) release through the channels, a process known as \(\text{Ca}^{2+}\) induced \(\text{Ca}^{2+}\) release (CICR, reviewed in Clapham, 1995; Hajnóczky, Hager and Thomas, 1999; Jackson and Thayer, 2006). Mitochondria located close to these release sites are able to initiate \(\text{Ca}^{2+}\) uptake and reduce the \([\text{Ca}^{2+}]\) near the receptors, thus inhibiting the positive feedback effect and reducing the extent of \(\text{Ca}^{2+}\) release from the ER (Hajnóczky, Hager and Thomas, 1999; Jackson and Thayer, 2006).

Store operated \(\text{Ca}^{2+}\) entry (SOCE) was a process initially thought to serve only as a homeostatic mechanism in which \(\text{Ca}^{2+}\) entry from the plasma membrane is promoted upon ER \(\text{Ca}^{2+}\) depletion in order to allow for replenishment of ER \(\text{Ca}^{2+}\) (reviewed in Hogan and Rao, 2016). However, SOCE has lately been shown to have signaling roles as well (reviewed in Putney et al, 2017; Sabourin et al, 2015). Furthermore, SOCE has also been shown to be impacted by both mitochondrial \(\text{Ca}^{2+}\) uptake through the MCU (Glitsch, Bakowski and Parekh, 2002; Samanta, Douglas and Parekh, 2009) and mitochondrial \(\text{Ca}^{2+}\) release through the NCLX (Ben-Kasus Nissim et al, 2017). In some types of polarized cells, mitochondrial \(\text{Ca}^{2+}\) uptake by spatially organized structures of mitochondria collectively referred to as “mitochondrial belts” has also been shown to spatially restrict the diffusion range of \(\text{Ca}^{2+}\) waves, leaving the \(\text{Ca}^{2+}\) wave restricted to specific regions in the cell (Thai et al, 2015; Tinel et al, 1999; reviewed in Petersen and Tepikin, 2008). Mitochondrial \(\text{Ca}^{2+}\) uptake is able to shape the patterns of several forms of \(\text{Ca}^{2+}\) signaling, thus interfering with responses the rest of the cell will have to them. It is also able to directly affect mitochondrial function in itself through the allosteric activation of Krebs cycle enzymes isocitrate dehydrogenase and oxoglutarate dehydrogenase in the mitochondrial matrix (reviewed in Denton, 2009), as well as indirectly activating pyruvate dehydrogenase (PDH) through the allosteric inhibition of
pyruvate dehydrogenase phosphatase (reviewed in Denton, 2009). Mitochondrial Ca^{2+} can also allosterically activate the mitochondrial enzyme glycerol phosphate dehydrogenase, activating the glycerophosphate shuttle and thus providing more NADH (or FADH_{2}, in the case of the glycerol phosphate dehydrogenase) to supply oxidative phosphorylation. This mechanism of NADH shuttling plays a secondary minor role when compared to the malate-aspartate shuttle, which is also positively regulated by Ca^{2+} at the level of the glutamate/aspartate carrier Aralar, a component of the shuttle with a regulatory site which does not require mitochondrial Ca^{2+} uptake since this Ca^{2+} binding site is exposed to the outer side of the inner mitochondrial membrane (reviewed in Denton, 2009; Gellerich et al, 2010). All of the processes described (upregulation of the Krebs cycle and NADH shuttling) increase oxidative phosphorylation at the level of increased reduced (NADH and FADH_{2} supply, an increase which can even be observed in vivo through NAD(P)H autofluorescence after stimulation with Ca^{2+} mobilizing agents which promote mitochondrial Ca^{2+} uptake (reviewed in Hajnóczky et al, 1995). While there is some evidence for Ca^{2+} regulation at the level of components of the ETC and ATP synthase contributing to the increase in oxidative phosphorylation promoted by mitochondrial Ca^{2+} [in fact, some of these evidences suggest that Cytochrome c oxidase activity is inhibited rather than stimulated by Ca^{2+} (Vygodina, Kirichenko and Kostantinov, 2013; Vygodina et al, 2017)], these results are not as widely accepted as the regulation at the level of increased substrate oxidation and NADH shuttling (reviewed in Bazil et al, 2016; Griffiths and Rutter, 2009; Tarasov, Griffiths and Rutter, 2012). These, along with conditions of increased energetic demand in which Ca^{2+} transfer to mitochondria usually occurs, seem to be responsible for increased oxidative phosphorylation rates observed in these contexts (Griffiths and Rutter, 2009).
Interestingly enough, this increased ATP supply by mitochondria not only helps the cell to match its occasional higher energetic demands but can also have regulatory roles. For instance, mitochondrial ATP production also directly regulates pancreatic beta cell function, where, unlike most cells, cytosolic ATP levels can vary greatly in proportion to substrate availability and are directly related to the extent of oxidative phosphorylation in these cells (reviewed in Tarasov, Dusonchet and Ashcroft, 2004). Higher cytosolic ATP levels result in the closure of ATP-sensitive K⁺ channels present in beta cell plasma membranes, leading to cell membrane depolarization and opening of voltage-dependent Ca²⁺ channels, initiating the process which ultimately leads to insulin secretion (reviewed in Tarasov, Dusonchet and Ashcroft, 2004; Fu, Gilbert and Liu, 2013). In fact, MCU knockout has already been shown to have an impact on the insulin-secreting capacity of pancreatic beta cells (Alam et al, 2012), suggesting a role for this type of regulation by mitochondrial Ca²⁺. The close positioning of mitochondria to sites rich in ATP consuming enzymes like the ER has also been suggested to positively regulate their activity by funneling ATP produced by oxidative phosphorylation (reviewed in Rieuseet, 2018). Indeed, reduced mitochondria-ER coupling or ATP/ADP exchange through the adenine nucleotide translocase (ANT) results in impaired protein folding and Ca²⁺ homeostasis in the ER (reviewed in Rieusset, 2018). Still regarding the regulation of mitochondrial function by Ca²⁺, several works have also shown a role for Ca²⁺ signals in the regulation of mitochondrial dynamics in the cell, with different Ca²⁺ signals either inducing mitochondrial fusion or fragmentation in a context-dependent manner (reviewed in Jeyaraju, Cisbani and Pellegrini, 2009). Calcium also regulates mitochondrial motility in the cell, with higher cytosolic [Ca²⁺] arresting mitochondrial movement machinery and
thus enabling mitochondria to accumulate at sites where cytosolic Ca\textsuperscript{2+} raises (reviewed in Yi, Weaver and Hajnóczky, 2004).

Mitochondrial dynamics, broadly speaking, consists of the processes by which mitochondria can change their shape, distribution and size inside the cell (reviewed in Tilokani et al, 2018). Individual mitochondria are able both to fuse their inner and outer membranes with neighboring mitochondria in order to form larger or even an interconnected network of mitochondria, and also undergo the opposite process, with larger, more connected mitochondria fragmenting into smaller and rounder mitochondria (reviewed in Tilokani et al, 2018). Mitochondria are also transported across the cell using the kinesin and dynein mechanisms, with smaller, more fragmented mitochondria, more readily transported (reviewed in Yi, Weaver and Hajnóczky, 2004). Smaller mitochondria are not only more easily transported, but also more easily targeted for mitophagy, a process in which dysfunctional mitochondria are eliminated and degraded in lysosomes (reviewed in Twig and Shirihai, 2011). On the other hand, fragmented mitochondria but are usually less efficient in synthetizing ATP, while larger, more interconnected mitochondria are usually more efficient. This may be attributed to sharing of intermediates and complementation of possible dysfunctional proteins of individual mitochondria by the contents of other mitochondria and to a higher number of cristae (reviewed in Liesa and Shirihai, 2013), which has been suggested to increase ATP-synthase activity by positioning them in a privileged position for proton pumping (Strauss et al, 2008). The fusion/fission balance of the mitochondrial network has also been shown to have an impact on stem cell fate in murine mesenchymal stem cells, although the precise mechanisms for that are still not clear (Forni et al, 2016). Transport and positioning of mitochondria in the cell has also been shown to be relevant for proper
cell physiology, especially mitochondrial transport across axons of neurons, which allow them to be positioned in regions of high ATP demand like near the synapses and allow for axon branching and proper function (reviewed in Lin and Sheng, 2015). Despite all these described effects, the impact of mitochondrial dynamics on cell physiology is a relatively recent research area, with many yet to be understood aspects. Under pathological conditions mitochondria participate in distinct mechanisms in situations of \( \text{Ca}^{2+} \) deregulation in which non-physiological levels of \( \text{Ca}^{2+} \) uptake occur and can thus greatly differ from more physiological contexts (reviewed in Kowaltowski, Castilho and Vercesi, 2001). As previously discussed, the negative-inside mitochondrial membrane allows for vast amounts of \( \text{Ca}^{2+} \) uptake. Since \( \text{Ca}^{2+} \) is a positive charged ion, its electrogenic uptake means that a drastically larger \([\text{Ca}^{2+}]\) in the mitochondrial matrix compared to the cytosolic \([\text{Ca}^{2+}]\) would be needed to reach thermodynamic equilibrium (reviewed in Santo-Domingo and Demareux, 2010). The relatively alkaline pH (compared to the cytoplasm) and presence of high phosphate concentrations in the mitochondrial matrix also allow for the formation of insoluble \( \text{Ca}^{2+}\)-phosphate complexes, which ultimately result in lower free \( \text{Ca}^{2+} \) concentrations in the mitochondrial matrix, further increasing the theoretical maximal \( \text{Ca}^{2+} \) buffering capacity of mitochondria (reviewed in Chinopoulos and Adam-Vizi, 2010). This capacity is highly relevant in \( \text{Ca}^{2+} \) deregulation contexts, in which extensive levels of mitochondrial \( \text{Ca}^{2+} \) uptake can occur (reviewed in Celsi et al, 2009; Tsujimoto and Shimizu, 2007), while in normal physiology they would normally not surpass a few micromolar at best (Bagur and Hajnóczky, 2017).

During the early stages of \( \text{Ca}^{2+} \) deregulation, this extensive process of mitochondrial \( \text{Ca}^{2+} \) uptake plays a cytoprotective role, allowing for mitochondria to act as \( \text{Ca}^{2+} \) sinks and helping the cell to cope with the excessive levels of cytosolic \( \text{Ca}^{2+} \) by
normalizing them (reviewed in Celsi et al, 2009). The problem with this role of mitochondria as a buffering system, however, lays in the fact that although the theoretical capacity for Ca\(^{2+}\) retention is large, it is in practice limited by the process of mitochondrial permeability transition (mPT). Upon reaching a certain level, mitochondrial matrix \([\text{Ca}^{2+}]\) induces the formation of a large unselective pore complex known as the mitochondrial permeability transition pore (mPTP, reviewed in Halestrap, 2009). mPT formation occurs by a mechanism which seems to be primarily linked to the oxidation of thiols in mitochondrial membrane proteins resulting from the \(\text{Ca}^{2+}\) induced oxidant production (reviewed in Kowaltowski, Castilho and Vercesi, 2001). Upon mPTP formation, not only the accumulated \(\text{Ca}^{2+}\) is released, but several other molecules are able to cross the inner mitochondrial membrane due to its unselective nature (up 1.5 kDa according to experimental measurements). This includes protons which are pumped outside the mitochondrial matrix by the ETC, completely collapsing the mitochondrial inner membrane potential. This not only compromises ATP production, but also contributes toward cellular ATP depletion through the reverse action of ATP synthase (in which it hydrolyzes ATP, instead of synthesizing it), which is thermodynamically more favorable after the collapse of membrane potential. This resulting ATP depletion leads to bioenergetic failure and cell death (reviewed in Halestrap, 2009). Opening of the mPTP can also promote extensive inner membrane swelling resulting in its expansion and unfolding of cristae, which can cause the rupture of the outer mitochondrial membrane, irreversibly damaging mitochondria while also promoting the release of pro apoptotic factors like cytochrome c (Tsujiimoto and Shimizu, 2007; reviewed in Halestrap, 2009). All these effects result in the second aspect of the dual role of mitochondrial \(\text{Ca}^{2+}\) uptake during \(\text{Ca}^{2+}\) deregulation: after mPT mitochondrial function is severely compromised,
which ultimately leads to cellular ATP depletion and cell death. Although some studies have questioned the relevance of this process for apoptosis-mediated cell death, its role in necrotic cell death is universally accepted (Nakagawa et al, 2005). Of note, some groups also suggest the presence of physiological reversible formation of the mPT pores in a low conductance state known as “flickering mode”, which could have physiological signaling roles. Even these groups, which suggest a role for mPT in physiology, agree that the formation of the mPTP is irreversible after a certain threshold is reached (reviewed in Mnatsakanyan et al, 2017). Interestingly enough, the [Ca^{2+}] threshold for mPTP formation is not fixed, with several positive and negative modulators and also varying from tissue to tissue (reviewed in Halestrap, 2009). Pharmacologically, the most well-known inhibitor of the mPTP is cyclosporin A (CsA), which exerts this effect by complexing with and sequestering the protein Cyclophilin D (CypD), a positive mPTP modulator. Treatment with CsA or CypD knockout/knockdown are both able to increase mitochondrial Ca^{2+} retention capacity (CRC), that is, the maximal amount of Ca^{2+} mitochondria can take before the onset of mPTP (Halestrap, 2009). The increase in CRC has been shown multiple times in several models to be cytoprotective in contexts of Ca^{2+} deregulation (reviewed in Halestrap, 2009; Rao, Carlson and Yan, 2014), making the results of basic research involving the regulation of the mPTP highly relevant for the treatment and prevention of pathological cell death conditions induced by mPT.

1.2 Modulation of mitochondrial calcium handling by caloric restriction

Our main goal in this thesis was to study possible changes in mitochondrial Ca^{2+} handling both in pathology and physiology under different interventions related to nutritional status, namely caloric restriction (CR) and short term fasting. Caloric
restriction (CR) is a dietary intervention known to promote several benefits for lab animals, like preventing or delaying the onset of diseases and complications associated with aging and oxidative imbalance, like insulin resistance (reviewed in Johnson et al, 2016), ischemia-reperfusion (reviewed in Rohrbach et al, 2014; Lempiäinen et al, 2013; Ran et al, 2015) injury and several neurodegenerative diseases (reviewed in Amigo and Kowaltowski, 2014). Notably, it has also been shown to be the most robust lifespan-extending intervention in a great variety of models, ranging from spiders, flies, worms and yeast to rodents and monkeys (reviewed in Heilbronn and Ravussin, 2003). Despite its well-known effects in preserving cell function and extending lifespan in these varied models, the precise mechanisms by which CR promotes its effects it is not completely understood. A large body of evidence, however, attributes several effects of CR to changes in cell metabolism and mitochondrial function, mainly through chances in mitochondrial oxidative phosphorylation capacity, coupling and production of oxidants (reviewed in Guarente, 2008).

Our group was the first to show that CR can also promote changes in mitochondrial Ca\textsuperscript{2+} handling (Amigo et al, 2017). Animals submitted to 4 months of CR had higher mitochondrial Ca\textsuperscript{2+} retention capacity in the brain and were highly protected against excitotoxicity, a condition in which neuronal Ca\textsuperscript{2+} overload and cell death occurs. The increased CRC in this model resulted from decreased activity of CyPD, which acts as a positive mPTP modulator (Amigo et al, 2017). Given the fact that many of these pathological conditions are related to cell death or dysfunction due to mitochondrial Ca\textsuperscript{2+} overload, and CR seems to be related to changes in mitochondrial function, we first sought to investigate the potential role of CR on mitochondrial Ca\textsuperscript{2+} handling in other tissues besides the brain.
Finally, nutrient availability has also been shown to be positively correlated with mitochondrial dynamics under many situations, with higher nutrient availability usually resulting in more fissioned, uncoupled mitochondria, while lower nutrient availability results in more fused and efficient mitochondria (reviewed in Liesa and Shirihai, 2013). Pathological conditions of nutrient overload like glucose/fatty acid overload in pancreatic beta cells also seem to induce excessive mitochondrial fragmentation, which ultimately results in cellular dysfunction. Prevention of mitochondrial fission is typically protective (Cerqueira et al, 2016), while exposing MEF cells to starvation overnight has been shown to induce mitochondrial fusion, which, when inhibited, results in mitochondrial depolarization and cell death (Rambold et al, 2011).

Unpublished results from our lab (shown in Chapter IV) have uncovered a regulation of mitochondrial Ca\(^{2+}\) handling by mitochondrial dynamics, with higher levels of mitochondrial fusion resulting in higher CRC and uptake rates, while more fragmented mitochondria show the opposite phenotype. These changes also regulate SOCE, which is known to be affected by mitochondrial Ca\(^{2+}\) uptake (Glitsch, Bakowski and Parekh, 2002; Samanta, Douglas and Parekh, 2009), showing that the modulation of Ca\(^{2+}\) handling is also relevant for physiological Ca\(^{2+}\) signaling processes. Although we did not use changes in nutrient availability to induce changes in mitochondrial dynamics, the fact that such an impact of mitochondrial dynamics on mitochondrial Ca\(^{2+}\) handling and Ca\(^{2+}\) signaling occurs uncovers a potential link between nutritional availability and mitochondrial morphology and Ca\(^{2+}\) handling under physiologically relevant contexts.
2. Goals

- Establish the effects of dietary interventions (caloric restriction and fasting) on mitochondrial function, focusing on mitochondrial Ca\(^{2+}\) handling.
- Study novel regulatory mechanisms for mitochondrial Ca\(^{2+}\) homeostasis
- Test the implications of possible functional changes in mitochondrial Ca\(^{2+}\) on pathological and physiological processes.
CHAPTER II - Caloric restriction protects livers from ischemia/reperfusion damage by preventing Ca^{2+}-induced mitochondrial permeability transition

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Preface and author contributions

This study had the goal of establishing if the results obtained in our previous work, which showed that CR promoted an increase in mitochondrial CRC in the brain, were also valid for other tissues like the liver. Mice were submitted to 4 months of CR and had their livers used for mitochondrial isolation, comparing mitochondrial Ca^{2+} handling from CR animals to a control *ad libitum* (eating freely) fed group. Our results not only show that CR animals did in fact shown increased liver mitochondrial CRC, but also faster uptake rates and protection against liver ischemia-reperfusion, a condition in which cell death can result from formation of the mPTP. Although the mechanism by which CR promoted these changes was distinct from the mechanisms observed for brain mitochondria, these results show that the regulation of mitochondrial Ca^{2+} handling by CR is not restricted only to the brain, possibly presenting a more general effect of the dietary intervention, which could help explain its cytoprotective effects in many pathological conditions.

Sergio L. Menezes-Filho, Alicia J. Kowaltowski and Ignacio Amigo designed the experiments. Ischemia-Reperfusion experiments were conducted by Natalie C. Ferreira and Edna Monteiro. HPLC for quantification of adenine nucleotides was conducted by Fernanda M. Prado and Marisa H.G. Medeiros. Isabella F.D. Pinto and Sayuri Miyamoto conducted the quantification of mitochondrial phospholipids by HPLC. All the remaining experiments were conducted by Sergio L. Menezes-Filho.
Graphical abstract

Ad Libitum

ATP/ADP ↑ Permeability Transition

Ischemia/Reperfusion Damage

Calcric Restriction

ATP/ADP ↓ Permeability Transition

Ischemia/Reperfusion Damage
Caloric restriction protects livers from ischemia/reperfusion damage by preventing Ca\textsuperscript{2+}–induced mitochondrial permeability transition

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**ABSTRACT**

Caloric restriction (CR) promotes lifespan extension and protects against many pathological conditions, including ischemia/reperfusion injury to the brain, heart and kidney. In the liver, ischemia/reperfusion damage is related to excessive mitochondrial Ca\textsuperscript{2+} accumulation, leading to the mitochondrial permeability transition. Indeed, liver mitochondria isolated from animals maintained on CR for 4 months were protected against permeability transition and capable of taking up Ca\textsuperscript{2+} at faster rates and in larger quantities. These changes were not related to modifications in mitochondrial respiratory activity, but rather to a higher proportion of ATP relative to ADP in CR liver mitochondria. Accordingly, both depletions of mitochondrial adenine nucleotides and loading mitochondria with exogenous ATP abolished the differences between CR and ad libitum (AL) fed groups. The prevention against permeability transition promoted by CR strongly protected against in vivo liver damage induced by ischemia/reperfusion. Overall, our results show that CR strongly protects the liver against ischemia/reperfusion and uncover a mechanism for this protection, through a yet undescribed diet-induced change in liver mitochondrial Ca\textsuperscript{2+} handling related to elevated intramitochondrial ATP.

1. Introduction

Mitochondrial permeability transition (mPT) is a form of inner mitochondrial membrane permeabilization that results in a loss of mitochondrial function and is promoted by excessive Ca\textsuperscript{2+} and oxidants [13]. If mPT is widespread, cell death may occur both due to metabolic failure and the activation of apoptotic pathways [13,25,45]. Interestingly, some studies indicate that susceptibility to mPT in different tissues increases with age [19,33,46], while others show that obesity-inducing high fat diets, which are associated with unhealthy aging, promote mPT [43]. This suggests that mPT may not only be determinant in the regulation of cellular energy metabolism and survival, but may also be regulated by age and dietary interventions.

Laboratory rodents are typically maintained sedentary and with ad libitum (AL) access to food, conditions that lead to obesity and associated morbidities such as non-alcoholic steatosis and insulin resistance [31]. Indeed, a 40% restriction in caloric intake without malnutrition (calorie restriction, CR) has been widely shown to increase laboratory animal lifespans and health spans [32,42,8]. Interestingly, we recently found that CR increases mitochondrial Ca\textsuperscript{2+} buffering capacity and prevents mPT in brain mitochondria, resulting in strong protection against excitotoxic damage [2], such as that which occurs in cerebral ischemia/reperfusion. These results indicate that one of the protective mechanisms of CR may be changes in mitochondrial Ca\textsuperscript{2+} homeostasis and the prevention of mPT.

We now hypothesized that CR may also regulate mitochondrial Ca\textsuperscript{2+} homeostasis in other organs. We approached this hypothesis by verifying the results of this dietary intervention on liver mitochondrial Ca\textsuperscript{2+} handling properties. In liver, CR is known to have a strong impact on many hallmarks of aging [29] such as age-related increases in the number of stellate cells and fibrosis [11,20]. Furthermore, liver mitochondrial Ca\textsuperscript{2+} handling has important regulatory properties and is a...
causal agent in damage promoted by ischemia/reperfusion [16,21,25,4]. Overall, our results demonstrate that changes in liver mitochondrial Ca$^{2+}$ handling are determinant in tissue protection promoted by CR.

2. Results

2.1. Caloric restriction increases mitochondrial calcium uptake and retention

Based on our recent results indicating that CR prevents mPT and increases Ca$^{2+}$ accumulation capacity in brain mitochondria [2], we decided to evaluate if changes in mitochondrial Ca$^{2+}$ uptake also occurred in the liver. Fig. 1A shows typical Ca$^{2+}$ uptake traces in isolated liver mitochondria from animals kept for 4 months on an ad libitum (AL, black line) or CR (gray line) diet. Ca$^{2+}$ concentrations in the extramitochondrial media were monitored using the fluorescent probe Ca$^{2+}$ Green 5N, and uptake of the ion is indicated by the decrease in fluorescence, since the probe does not cross mitochondrial membranes. Ca$^{2+}$ additions were made where indicated by the arrows, and Ca$^{2+}$ uptake was followed until the maximum uptake capacity was reached and Ca$^{2+}$ induced mPT occurred (as indicated by the upward deflection of the curve, indicative of ion release by mitochondria). We consistently found that isolated mitochondrial preparations from CR animals had approximately 70% higher Ca$^{2+}$ retention capacity compared to AL mitochondria (quantified in Fig. 1C, p < 0.0001). We then measured Ca$^{2+}$ retention capacity in preparations pre-treated with the mPT inhibitor cyclosporin A (CsA; Fig. 1B, quantified in Fig. 1C). By inhibiting mPT, CsA promotes a large increase in Ca$^{2+}$ retention capacity (note cut in the Y axis of Fig. 1C), and also leads to equal accumulation in CR and AL samples. This indicates that AL mitochondria have increased mPT susceptibility, undergoing this form of inner membrane permeabilization at lower Ca$^{2+}$ loads.

The same traces used to measure Ca$^{2+}$ retention capacity can be used to measure Ca$^{2+}$ uptake rates, or the initial speed in which mitochondria remove the ion from the extramitochondrial media. Interestingly, CR mitochondria not only took up higher Ca$^{2+}$ quantities, but also did so at faster rates (Fig. 1D, quantified in Fig. 1F, p < 0.05). The presence of CsA (Fig. 1E) did not significantly change these rates nor equal Ca$^{2+}$ uptake speed in CR and AL mitochondria (Fig. 1F, p < 0.05). This suggests that, in addition to being more resistant to the mPT, which typically occurs under pathological conditions with Ca$^{2+}$ overload, liver CR mitochondria present changes in functionality that increase the ability to buffer Ca$^{2+}$ acutely, under physiologically-relevant conditions.

2.2. CR does not alter mitochondrial respiration or coupling, but changes the redox state

Mitochondrial Ca$^{2+}$ uptake is driven by the mitochondrial inner membrane potential, which is maintained by the electron transport chain. Based on the results showing that Ca$^{2+}$ uptake rates were altered (Fig. 1D), we hypothesized that mitochondrial respiratory function and coupling could be modified by CR [35], explaining the enhanced Ca$^{2+}$ uptake ability. Interestingly, respiratory rate measurements (typical traces are shown in Fig. 2A, quantified in Fig. 2B) showed no difference in basal levels, oxygen consumption stimulated by the presence of ADP (state 3), inhibited by the ATP synthase inhibitor oligomycin (state 4) or maximized by the presence of the uncoupler CCCP (maximal). Furthermore, respiratory control ratios (Fig. 2C) were equal, indicating that no change in mitochondrial coupling and inner membrane integrity was promoted by CR. Indeed, NADH levels (Fig. 2E) and overall membrane lipid composition (Fig. S1) were also unchanged by the dietary intervention. Overall, these results show that changes in electron transport function and/or mitochondrial coupling are not responsible for the increase in Ca$^{2+}$ uptake promoted by CR observed in Fig. 1.

Interestingly, despite the fact that respiratory function and coupling were identical between AL and CR groups, mitochondrial H$_2$O$_2$ production was decreased in the CR group (p < 0.05, Fig. 2D). Caloric restriction is often associated with lower mitochondrial oxidant production [39], and this could play a role in the decreased susceptibility.
of CR mitochondria to mPT since oxidation of protein thiols in the inner mitochondrial membrane plays a seminal role in the onset of permeability transition [24]. Mitochondrial NADPH levels are also determinant in regulating the mPT [13,48,50] by maintaining the redox state and antioxidant capacity, and were thus measured in our samples. Interestingly, AL mitochondria presented significantly higher NADPH levels (Fig. 2F, p < 0.05). A possible explanation is that NADPH increases occur as a response to the higher levels of oxidants generated by AL mitochondria (Fig. 2D), as a compensatory mechanism. Since NADPH is an mPT inhibitor, this result eliminates the possibility that the changes in Ca^{2+} uptake observed in Fig. 1 were due to enhanced NADPH availability in CR mitochondria. Furthermore, changes in NADPH levels and oxidant generation would be expected to alter maximal Ca^{2+} uptake capacity [50], but not uptake rates, as is observed in CR, suggesting that these effects are promoted by factors other than redox state.

2.3. CR increases mitochondrial ATP levels, leading to enhanced Ca^{2+} uptake

Mitochondrial adenine nucleotides are also important Ca^{2+} uptake regulators, acting both by complexing intramitochondrial Ca^{2+} with their negatively charged phosphate groups [18] and preventing mPT pore opening [17,47]. To verify if changes in adenine nucleotide levels could be responsible for the changes in Ca^{2+} uptake promoted by CR, we measured Ca^{2+} uptake in the presence of 2 mM ATP (Fig. 3A, quantified in Fig. 3B) or 2 mM ADP (Fig. 3B). As expected, the presence of adenine nucleotides increased Ca^{2+} uptake capacity (Fig. 3B).

Interestingly while presence of ATP equaled maximal Ca^{2+} uptake (Figs. 3A, B) and uptake rates (Fig. 3C, quantified in 3D) in CR and AL samples, experiments conducted in the presence of ADP still showed a higher Ca^{2+} retention capacity for CR mitochondria (Fig. 3B, p < 0.05; unfortunately Ca^{2+} uptake rates with ADP could not be accurately determined due to the non-linear behavior of Ca^{2+} uptake kinetics).

These results suggest that mitochondrial ATP levels are probably involved in the enhancement of Ca^{2+} uptake promoted by CR. Indeed, the presence of an extra negative charge in ATP relative to ADP makes its affinity for Ca^{2+} roughly 10 times higher [18], which could increase uptake rates and retention capacity. For example, exchanging ADP for ATP promotes an increase in free mitochondrial Ca^{2+} independently of changes in matrix volume or Ca^{2+} uptake [18]. To verify if changes in ATP/ADP levels in mitochondria occurred in CR, we measured adenine nucleotide levels in mitochondrial preparations using HPLC-MS/MS. Due to the significant loss in adenine nucleotides which occurs with processing and storage, samples for this analysis were paired as AL and CR mitochondria isolated on the same day and processed in parallel. Although we did not find a difference in total adenine nucleotide content (not shown), we did see a significant increase in the relative content of ATP (Fig. 3E, p < 0.05). These increases in relative matrix ATP concentrations may explain the enhanced Ca^{2+} buffering by CR mitochondria.

If changes in mitochondrial ATP levels are the cause for enhanced Ca^{2+} uptake rates in CR, depleting mitochondrial ATP should eliminate the difference between AL and CR samples. Indeed, when intramitochondrial ATP was depleted by incubation with pyrophosphate [1], Ca^{2+} uptake levels were quite low, but equal in AL and CR.
Fig. 3. ATP normalizes differences in Ca\textsuperscript{2+} uptake promoted by CR. Ca\textsuperscript{2+} uptake capacity (Panels A and B) and rates (Panels C and D) were determined in the presence of 2 mM ATP (A, B) or 2 mM ADP (B), under conditions described for Fig. 1. In Panel E, ATP and ADP contents in isolated mitochondria were determined as described in the Experimental section. * p < 0.05, paired to AL controls.

Fig. 4. ATP depletion and reloading makes Ca\textsuperscript{2+} uptake equal in AL and CR samples. Ca\textsuperscript{2+} uptake capacity (A, B, D and E) and rates (C, F) were measured in ATP-depleted (A, C) and ATP-reloaded (B, C, E and F) mitochondria, produced as described in the Experimental section, under conditions similar to those described in Fig. 1.
mitochondria (Fig. 4A, quantified in Fig. 4D). Furthermore, reloading mitochondria with exogenous ATP [1] increased Ca\(^{2+}\) uptake capacity (Figs. 4B and F) and rates (Figs. 4C and F) to equal levels in AL and CR samples. Thus, changes in intramitochondrial ATP levels are determinant for the enhanced Ca\(^{2+}\) uptake capacity observed in CR mitochondria.

We sought to clarify the mechanism in which ATP levels in CR mitochondria increase. Since no changes were observed in mitochondrial respiration or coupling (Fig. 2A-C) (indicating no changes in the capacity of mitochondria to synthesize or transport ATP), SCAvMC-3, an ATP-Mg/Pi carrier which can promote changes adenine nucleotide content in the fasted liver by transporting ATP into the mitochondria is a possible mechanism. Indeed, fasting is known to activate SCAvMC-3 [1]. However, we did not observe any changes in SCAvMC-3 levels (Fig. S2). Lipid composition, which can affect transporter activity [38] was also overtly unaltered by CR (Fig. S1). Thus, the mechanism in which this dietary intervention promotes mitochondrial ATP increases in the liver remains to be determined.

2.4. Caloric restriction prevents liver ischemia/reperfusion injury

Liver damage by ischemia/reperfusion is related to intracellular Ca\(^{2+}\) deregulation, with excessive mitochondrial Ca\(^{2+}\) uptake, mPT and organelar dysfunction [45]. Indeed, genetic or pharmacological inhibition of mPT protects against ischemia/reperfusion damage [37,4]. Interestingly, although CR has been shown to protect kidneys, brains and hearts against ischemic damage [26,40,41], only the effects of acute fasting or protein restriction were tested in liver to date [34,36].

We thus sought to verify if the enhanced resistance to mPT promoted by CR was associated with a prevention of ischemia/reperfusion damage. AL or CR mice were fasted overnight (to normalize acute nutritional status) and submitted to 40 min ischemia followed by 1 h reperfusion. The livers were then collected for histological analysis to evaluate cell death (Figs. 5A and B) and inflammation (Figs. 5C and D) in the tissue. No overt difference in inflammation was noted in the histologies, but the CR group was very strongly protected against injury induced by ischemia/reperfusion, showing very low infarct damage (< 1% of the total area), while AL mice showed a significantly higher area of tissue damage (~25%, data from the histological analysis was quantified in Fig. 5E, p < 0.05). Confirming that the ischemia/reperfusion damage observed was mPT-dependent, Ca\(^{2+}\) completely prevented the lesion, leading to undetectable infarct areas (Fig. 1E). Overall, this result shows that CR very strongly protects the liver against ischemia/reperfusion injury by preventing mPT.

3. Discussion

In laboratory rodents, CR prevents obesity induced by overeating and sedentary living. This dietary intervention is well documented to extend lifespan and delay age-related diseases, as well as improve stress responses [29]. The mechanisms through which these beneficial effects occur have long been investigated, and involve, predictably, changes in energy metabolism and mitochondria. To date, CR has been shown to impact upon mitochondrial mass [28], morphology [10], respiratory function [12,35], coupling [3], oxidant production [14], oxidative damage [14], mtDNA integrity [12] and turnover (López-Lbch et al., 2015). Effects vary in different tissues, animal models, diets and time points, but generally show a preservation of mitochondrial metabolic function over time.

Another important metabolic regulator is intramitochondrial Ca\(^{2+}\), a key determinant of ATP production [15], a role recognized since the 1980s despite the fact that the molecular identity of the mitochondrial Ca\(^{2+}\) uniporter was only uncovered in 2011 [6]. Interestingly, recent findings suggest mitochondrial Ca\(^{2+}\) homeostasis may not only regulate energy metabolism, but also be modified by dietary interventions: high fat diets promote mPT in the heart [27] and skeletal muscle [43], while ketogenic diets appear to prevent mPT in brain mitochondria [22]. By directly measuring Ca\(^{2+}\) uptake from CR versus AL animals, we have recently found that CR very significantly increases mitochondrial Ca\(^{2+}\) uptake capacity and inhibits mPT in isolated brain mitochondria [2].

Our results in this manuscript now add to this finding by indicating that modulation of Ca\(^{2+}\) uptake promoted by CR is not limited to the brain, occurring also in the liver (Fig. 1). Furthermore, we find that CR-mediated mitochondrial Ca\(^{2+}\) modulation in the liver involves not only mPT prevention, but also an increase in intramitochondrial ATP...
(Fig. 3), resulting in faster and larger Ca\(^{2+}\) buffering capacity (Figs. 1 and 4). Our results are compatible with the finding that mitochondrial adenine nucleotide content in the liver can be hormonally regulated and determinant for Ca\(^{2+}\) retention capacity [1].

How CR promotes alterations in ATP/ADP ratios is still a matter for further investigation since no changes were seen in mitochondrial respiration or coupling (Fig. 2A-C) and levels of ATP-Mg/Pi carrier SCAMC-3, the protein responsible for adenine nucleotide content changes during fasting [1], were unaltered by CR (Fig. S2). Alterations in membrane lipid composition, which can alter the activity of mitochondrial membrane proteins [38], were also not observed (Fig. S1), although there could still be changes in the distribution of these phospholipids between the inner and outer mitochondrial membrane, as has been shown for cardiolipin [30].

Surprisingly, even though we observed increased \(\text{H}_2\text{O}_2\) production and NADPH contents in AL mitochondria, both of which influence mPT formation [24], these redox changes do not appear to be determinant for the differences between AL and CR mitochondrial mPT susceptibility and Ca\(^{2+}\) handling, since these were abolished by equal mitochondrial ATP contents in AL and CR samples. Since \(\text{H}_2\text{O}_2\) acts as a positive mPT modulator by promoting oxidative imbalance and membrane protein thiol oxidation while NADPH acts as a negative modulator by enhancing antioxidant systems, it is possible that these effects counteract each other. In fact, increased NADPH levels could be the result of stress responses triggered by the increased \(\text{H}_2\text{O}_2\) levels.

Thus, our results indicate that CR increases Ca\(^{2+}\) uptake and prevents mPT by changing mitochondrial ATP levels. This set of findings is, to our knowledge, the first direct evidence that changes in mitochondrial Ca\(^{2+}\) homeostasis may be a regulatory target of CR in its liver protective effects. This is of importance since significant CR-delayed and age-related diseases involve failures in mitochondrial Ca\(^{2+}\) handling [7]. Furthermore, ischemia/reperfusion damage to various tissues occurs secondary to mPT [21, 44], and increases with age.

In ischemia/reperfusion, mitochondrial Ca\(^{2+}\) uptake can momentarily buffer the sudden increases in cytosolic Ca\(^{2+}\) [30]. However, when a certain threshold is surpassed, mPT is induced and oxidative phosphorylation fails, contributing toward cell death [21]. The importance of mPT in ischemia/reperfusion is demonstrated by the protective effects of non-immunosuppressive mPT inhibitors on ischemic mouse livers [45]. In fact, mPT in the liver is involved in both necrotic and apoptotic ischemic cell death (Malhi et al., 2006). We find that CR not only increases mitochondrial Ca\(^{2+}\) buffering and the threshold for mPT induction (Fig. 1A-C), but also very substantially protects against ischemia/reperfusion damage (Fig. 5). Previous studies have shown that fasting also protects against liver ischemia/reperfusion, suggesting a “CR like” adaptation based on gene expression patterns found in the fasted liver (36, Mitchell et al., 2016). While the term “dietary restriction” was used in these studies, it is important to note that the protocol they used consisted of a very short fasting period (up to 3 days) or one week of protein restriction, which is very different from our chronic, lifespan-enhancing, CR protocols [8]. Importantly, all mice used in our study were fasted prior to the experiments, indicating that the protective CR effects observed are additive to those of acute fasting.

Overall, our results show a new protective effect of CR, the prevention of ischemia/reperfusion damage in the liver, and a new mechanism in which this protective effect occurs, namely modulation of mitochondrial adenine nucleotides resulting in faster and higher capacity Ca\(^{2+}\) uptake, preventing mPT. Given the importance of mitochondrial Ca\(^{2+}\) and adenine nucleotide content in liver physiology, we hope our findings will open new investigative lines involving mechanisms in which CR leads to longer, healthier lives.

4. Experimental procedures

4.1. Animals and calorific restriction

The use of male Swiss mice was approved by the local animal care and use committee and followed National Institutes of Health guidelines for humane treatment of animals. CR was initiated at 8 weeks after 1 week monitoring ad libitum (AL) chow consumption. The animals were separated in two groups, a control AL group eating at will and a CR group, eating the equivalent to 60% of the AL consumption of a micronutrient-supplemented chow [9], given to the mice at 11:00 a.m. The diet was maintained for 4 months and adjusted weekly based on AL food consumption. Four months of diet was adopted since this is the minimal time point in which we find consistent changes between AL and CR animals [2]. Both the standard AL and supplemented CR chow were prepared by Rhoster (Campinas, SP, Brazil). Experiments were done in parallel (1 AL and 1 CR animal per day) with mice fasted overnight, starting at 5 p.m. Results include 3–10 animals per group.

4.2. Mitochondrial isolation

Mitochondria were isolated by differential centrifugation from the livers of mice fasted overnight [44] and resuspended in 300 μL of experimental buffer (75 mM N-mannitol, 25 mM sucrose, 5 mM HKHP4, 20 mM Tris-HCl, 100 mM KCl and 0.1% BSA) containing 1 μM Ca\(^{2+}\) Green 5 N, 1 mM succinate, 2 μM rotenone and 1 μM MgCl2. Ca\(^{2+}\) Green fluorescence was measured at 506 nm excitation and 532 emission using a F2500 Hitachi Fluorimeter at 30 °C with continuous stirring. Where indicated, 5 μM Ca\(^{2+}\), 2 mM ATP or 2 mM ADP were added to the buffer before the addition of mitochondria. After 3 min, 10 μM Ca\(^{2+}\) additions were made every 90 s. When ATP or ADP were present, 75 μM Ca\(^{2+}\) additions were used due to the increase in Ca\(^{2+}\) retention capacity (CR).

Using Ca\(^{2+}\) uptake traces, fluorescence values (F) were converted into [Ca\(^{2+}\)]\(_{i}\) by using the formula [Ca\(^{2+}\)]\(_{i}\) = KD (F – F_{min})/(F_{max} – F). The KD value was experimentally estimated as the value which corresponds to a 10 μM increase in [Ca\(^{2+}\)]\(_{i}\) after each addition, since the presence of Mg\(^{2+}\) interferes with the theoretical KD. F_{max} was obtained by adding 100 μL of a saturated Ca\(^{2+}\) solution until no increase in fluorescence was observed, and F_{min} was estimated by doing the same with an EGTA solution. Ca\(^{2+}\) uptake rates were determined as the slope of the linear part of the Ca\(^{2+}\) uptake curve observed after the first Ca\(^{2+}\) addition (nmol Ca\(^{2+}\)/mg protein). s. Calcium retention capacity was calculated as the total amount of Ca\(^{2+}\) taken up by mitochondria before the onset of mPT (nmol Ca\(^{2+}\)/mg protein), as seen by the lack of further Ca\(^{2+}\) accumulation and increase in Ca\(^{2+}\) Green fluorescence due to Ca\(^{2+}\) release from mitochondria.

4.4. Oxygen consumption in isolated mitochondria

Oxygen consumption was measured using a high-resolution Oroboros oxygraph. Mitochondria (500 μg/2 mL) were suspended in the same experimental medium used in Ca\(^{2+}\) uptake assays in the absence of Ca\(^{2+}\) Green and Mg\(^{2+}\). State 3 respiration was measured by adding 20 mM ADP, State 4 by adding 1 μM oligomycin and maximal respiration was promoted by successive additions of 1 μM CCCP until maximal O2 consumption was observed. Respiratory control ratios (RCR) were determined as State 3/State 4 rates.
4.5. Hydrogen peroxide release from isolated mitochondria

Isolated mitochondria (250 μg) were incubated in 2 mL of the same experimental buffer as for the Ca^{2+} uptake assays, but without Ca^{2+} Green and with the addition of 50 μM Amplex Red and 2 U of Horseradish Peroxidase (HRP). H_{2}O_{2} production was measured by monitoring the increase in Amplex Red fluorescence at 563 nm excitation and 587 nm emission using a F2500 Hitachi Fluorimeter at 30 °C. A calibration curve was constructed by adding known quantities of H_{2}O_{2} to the same experimental conditions in the absence of mitochondria.

4.6. NADH and NADPH quantifications

Isolated mitochondria (500 μg) were incubated in 2 mL Ca^{2+} uptake experimental buffer without Ca^{2+} Green. NAD(P)H fluorescence was monitored at 366 excitation and 450 emission using a F2500 Hitachi Fluorimeter with constant stirring at 30 °C. After stabilization of the basal fluorescence signal, 1 mM acetoacetate was added in order to promote complete NADH oxidation through its conversion to β-hydroxybutyrate. After the second stabilization of the signal, two additions of 100 μM t-butyl hydroperoxide were made in order to completely oxidize the remaining NADPH. NAD(P)H concentrations were determined based on a calibration curve with known NADH concentrations. The remaining fluorescence after t-butyl hydroperoxide additions was considered background fluorescence. NADH and NADPH concentrations were estimated by the decrease in fluorescence after the addition of acetoacetate and t-butyl hydroperoxide, respectively.

4.7. Adenine nucleotide extraction and HPLC-MS/MS analysis

Adenine nucleotides were extracted from mitochondria with perchloric acid as described in Vives-Bauza et al. [49]. Freshly isolated mitochondria were treated with 0.4 M perchloric acid in order to inactive mitochondrial ATPases and precipitate proteins. The solution was kept 30 min on ice and centrifuged at 18,000 g. Supernatants were transferred to another tube and treated with 10% volume 4 M K_{2}CO_{3} to neutralize the perchloric acid. The solution was kept 10 min over ice and 1 h at −80 °C, and centrifuged at 18,000 g again after thawing to promote salt precipitation, which was discarded by transferring the supernatant to another recipient. Supernatants were stored at −80 °C until HPLC-MS/MS analysis.

For HPLC-MS/MS analysis, each 5 μL sample obtained in the extraction process was diluted in 1000 μL of 10 mM tributyl amine/15 mM acetic acid, homogenized (10 min, 4 °C, 1400 rpm) and aliquoted. 1.7 μL of 30 μM 13C_{10}, 15N_{5}-ATP (internal standard) was added to 98.3 μL of the sample and 10 μL of the resulting solution were injected into the HPLC-MS/MS system. Sample analysis was carried out using an 1200 Agilent HPLC system (Waldbom, Germany) coupled to a 6500 QTrap mass spectrometer (SCIEX, Concord Canada) with an electrospray source. HPLC separation proceeded in a 100 × 2.0 mm Luna 2.5 μm C18(2)-HST column (Phenomenex) with a flow rate of 250 μL/min. The mobile phase was 10 mM tributyl amine/15 mM acetic acid (A) and acetonitrile (B). The gradient started with 25–98% B during 0.95 min, followed by 9 min 98% B, returned to 25% B for 2 min and a re-equilibration step was performed for 20 min with 25% B. The column was maintained at 40 °C during analysis and samples were kept at 4 °C. For analysis, a valve to the direct flow rate from the HPLC column to the mass spectrometer was used from 1.0 to 5.0 min.

For mass spectrometry in tandem (MS/MS) analysis, samples were analyzed in the negative ion mode using Selected Reaction Monitoring. The collision activated dissociation gas flow was set as medium and dwell time for all transitions was optimized to 80 ms. For ATP, ADP and AMP quantification, three different calibration curves were prepared with the internal standard, 13C_{10}, 15N_{5}-ATP at a final concentration of 0.50 μM. ATP, ADP and AMP calibration curves ranged from 0.025 to 0.500 μM, 0.010–0.400 μM and 0.010–0.200 μM, respectively. For example, the ATP calibration curve was constructed plotting the ratio of the ATP area by the 13C_{10}, 15N_{5}-ATP area versus the ATP concentration. After quantification, readings were normalized by protein concentrations.

4.8. Mitochondrial adenine nucleotide depletion and ATP loading

Adenine nucleotides were depleted by ressusspending isolated mitochondria in 30 mL experimental buffer containing 1 mM EGTA and 0.5 mM EDTA and incubating at 30 °C for 5 min with 2 mM pyrophosphate [1]. The suspension was then centrifuged at 10,000 g, resuspended and washed twice in order to remove pyrophosphate. ATP loading was conducted by ressusspending depleted mitochondria in 30 mL experimental medium containing 10 μM Ca^{2+} and 4 mM ATP and incubating for 10 min at 30 °C [1]. Suspensions were also washed and centrifuged twice at 10,000 g in order to remove external ATP.

4.9. Liver ischemia/reperfusion

Animals fasted overnight received an intramuscular injection of atropine (0.04 mg/kg) 10 min before induction of anesthesia, followed by the intramuscular administration of xylazine (10 mg/kg) and ketamine (70 mg/kg). An AL and CR group (indicated in Fig. 1) received a 50 mg/kg intraperitoneal injection of cyclosporin A (CsA, Sandimmun brand) just prior to anesthesia. Animals were considered in an anesthetized state when cornea-eyelid and posterior paw reflexes were lost. Trichotomy of the abdominal region and cleaning with alcohol 70% were then conducted. Physiological 0.9% saline was administered at a volume corresponding to 80 mL/kg animal weight. Animals were fixed to the surgical stand over a thermal mattress at 38 °C during the whole procedure. A surgical microscope at a magnification of 6–16 times was used. The hepatic pedicle was occluded above the emergence of the lateral right side branches, cautade lope and papillary process with a microsurgical vascular clamp for 40 min, followed by 1 h reperfusion. The abdominal wall was sutured with nylon 7-0 thread in two planes: peritone-muscle-aponeurotic and skin. After the procedure, animals were maintained in cages with water and chow ad libitum. After 1 h, animals were anesthetized again if needed and re-operated for surgical removal of the liver for histological analysis, followed by euthanasia by exsanguination under anesthesia.

4.10. Histological analysis

Part of the left lateral lobule of the mice was fixed in 10% formalin solution and submitted to histological processing after treatment with paraffin wax. The paraffin blocks were cut 4 μm thick using a RM2235 microtome (Leica, Germany) and stained with H/E. The samples were analyzed blindly using an Imager. A2 microscope (Zeiss, Germany) according to parameters established by Kleiner et al. [23]. Images were acquired using an AxioCam MRC camera (Zeiss, Germany) coupled to the microscope and analyzed with AxioVision LE software (Zeiss, Germany).

4.11. Statistical analysis

All data sets were analyzed using a two tailed unpaired student T test, except for adenine nucleotide contents and ATP/ADP ratios, in which a two tailed paired student T tests were used due to day-to-day variation in paired samples, probably due to ATP degradation during the paired processing. A p-value of 0.05 was considered statistically significant.

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Author contributions

SLMF, IA, SM and AJK designed the study; all authors designed experiments; SLMF, IA, FMP, MKK, NCF, IFDP and ESM conducted the experiments and analyzed data; SLMF and AJK wrote the manuscript, which was corrected by all authors.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2017.06.013.

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Supplementary Experimental Procedures

Western Blotting

Isolated mitochondrial samples were resolved by SDS-PAGE on 10% polyacrylamide gels, transferred to nitrocellulose membranes and blocked with 5% BSA. Primary polyclonal antibodies against SCaMC-3 made in rabbit were obtained as a kind gift from professors Araceli del Arco and Jorgina Satrústeui and used at a dilution of 1:2000. Rabbit polyclonal Anti-VDAC was purchased from Abcam (Ab15895) and used at a dilution of 1:5000. Secondary donkey anti-rabbit fluorescent antibodies were purchased from LI-COR (926-68073) and used at 1:10000. Images were obtained using an Odyssey imaging system and bands were quantified using the ImageJ software.

Mitochondrial Lipid Quantification

Non-targeted lipidomic analysis of major phospholipids of purified mitochondria was performed by liquid chromatography coupled to mass spectrometry (LC-MS). Prior to lipid extraction, a mixture of lipid internal standards (Table S1) was added to the samples for semi-quantification of reported lipid molecular species. Lipid extraction was performed according to the method established by Matyash et al. (2008). Briefly, 40 μL of purified mitochondria were mixed with 260 μL of ice-cold methanol and 40 μL of internal standards (10 - 20 ng/μL). After thoroughly vortexing for 10 s, 1 mL of methyl tert-butyl ether (MTBE) was added to the mixture, which was stirred for 1 h at room temperature. Next, 300 μL of water was added to the mixture, followed by vortexing 10 s and resting in an ice bath for 10 min. After centrifugation at 10,000 g for 10 min at 4°C, the supernatant containing the total lipid extract (TLE) was transferred to a new tube and dried under N2 gas.
The TLE were analyzed by ESI-TOFMS (Triple TOF 6600, Sciex, Concord, US) interfaced with a high-performance LC (UHPLC Nexera, Shimadzu, Kyoto, Japan). Dried TLE were re-dissolved in 100 μL of isopropanol and the injection volume was set at 2 μL. TLE were loaded into a CORTECS® (UPLC® C18 column, 1.6 μm, 2.1 mm i.d. x 100 mm) with a flow rate of 0.2 mL/min and the oven temperature maintained at 35°C. For reverse-phase liquid chromatography (RPLC), mobile phase A consisted of water/acetonitrile (60:40), while mobile phase B composed of isopropanol/acetonitrile/water (88:10:2). Mobile phases A and B contained ammonium acetate at a final concentration of 10 mM for experiments performed in negative ionization mode. The linear gradient during RPLC was as follows: from 40 to 100% B over the first 10 min, hold at 100% B from 10-12 min, decreased from 100 to 40% B during 12-13 min, and hold at 40% B from 13-20 min.

The MS was operated in negative ionization mode and the scan range set at a mass-to-charge ratio of 200-2000 Da. Data for lipid molecular species identification and quantification was obtained by Information Dependent Acquisition (IDA®). Data acquisition was performed with a period cycle time of 1.05 s with 100 ms acquisition time for MS1 scan and 25 ms acquisition time to obtain the top 36 precursor ions. Data acquisition was performed using Analyst® 1.7.1 with an ion spray voltage of -4.5 kV and 5.5 kV (for negative and positive modes, respectively) and the cone voltage at +/- 80 V. The curtain gas was set at 25 psi, nebulizer and heater gases at 45 psi and interface heater of 450°C. MS/MS data was analyzed with PeakView®, and lipid molecular species were identified by an in-house manufactured Excel-based macro. Lipid quantification was performed with MultiQuant®, where peak areas of precursor ions were normalized to those of the internal standards (Table S1). All of the lipid internal standards were purchased from Avanti Polar Lipids, Inc., Alabaster, AL.
Supplementary Tables

Table 1. Lipid internal standards.

| Internal standard                                                                 | Lipid classes | Concentration (ng/μL) |
|----------------------------------------------------------------------------------|---------------|-----------------------|
| 1,2-diheptadecanoyl-sn-glycero-3-phosphocholine (PC 17:0/17:0)                  | PC            | 19.8                  |
| 1,2-diheptadecanoyl-sn-glycero-3-phosphoethanolamine (PE 17:0/17:0)             | PE            | 19.8                  |
| 1,1',2,2'-tetramyristoyl cardiolipin (CL 14:0)_{4}                               | CL and PI     | 19.8                  |
| 1,2-diheptadecanoyl-sn-glycero-3-phosphoglycerol (PG 17:0/17:0)                 | PG            | 20                    |
| 1-heptadecanoyl-2-hydroxy-sn-glycero-3-phosphocholine (LysoPC 17:0)              | Lyso          | 19.8                  |
| N-heptadecanoyl-D-erythro-sphingosylphosphorylcholine (17:0 SM)                  | SM            | 15.8                  |
| N-decanoyl-D-erythro-sphingosine (10:0 Cer)                                      | Cer           | 10                    |
Supplementary Figures

Fig. S1: Total mitochondrial membrane phospholipid composition is not altered by CR. Lipidomic analysis was performed in negative mode by UPLC-ESI-Q-TOF-MS. Data are presented as relative abundance (% in average ± SEM, n = 3). AL, Ad libitum group; CR, caloric restriction group; CL, cardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; SM sphingomyelin; Cer, ceramide; Others: lysoPC and pPE, PE-plasmalogen. No statistically significant differences were observed between AL and CR samples (p > 0.05).
**Fig. S2**: CR does not alter the amount of SCaMC-3 in liver mitochondria. The abundance of the ATP-Mg/Pi Carrier SCaMC-3 in AL and CR mitochondria was compared by western blot, using VDAC as a loading control. (A) Representative bands. (B) Quantification. No statistically significant differences were observed between AL and CR samples (p > 0.05).

**Reference**

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CHAPTER III - Fasting promotes functional changes in liver mitochondria

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Preface and author contributions

This study had the goal to determine the effects of fasting on mitochondrial bioenergetics and Ca\(^{2+}\) handling in the liver. Overnight fasting is widely adopted in the literature and was suggested to improve the quality of isolated mitochondrial preparations. Experiments administering glucagon to live animals and cell culture models also supported that idea, given the stimulation of mitochondrial oxygen consumption and CRC observed. Preliminary results from our lab suggested, however, that liver mitochondrial preparations obtained from mice fasted overnight were actually less functional than mitochondria from fed animals. Given the fact that we found no study in the literature had previously addressed the effect of fasting itself on mitochondrial function, we decided to design this study to properly address that question. The results shown here demonstrate that mice submitted to both overnight (15 h) and short term (4 h) fasting present less functional liver mitochondria, with these effects not being attributed to contamination or impurity of the isolated mitochondrial samples or torpor-induced metabolic suppression.

Ignacio Amigo, Sergio L. Menezes Filho and Alicia J. Kowaltowski designed the experiments. Luis A. Luévano-Martinzes performed the quantification of cardiolipin by thin layer chromatography. Sergio L. Menezes Filho and Ignacio Amigo conducted the remaining experiments.
Fasting promotes functional changes in liver mitochondria

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A B S T R A C T

Overnight fasting of rodents is commonly adopted in protocols to obtain isolated liver mitochondria, but the effects of fasting itself on mitochondrial function are poorly characterized. In this study we show that overnight fasting (15 h) promotes a shift in the liver mitochondrial bioenergetic profile, with a reduction in ADP-stimulated and maximal respiration, lower membrane potentials and lower resistance to Ca²⁺-induced mitochondrial permeability transition. Short term fasting (4 h) promoted similar changes, suggesting that this is a physiological shift in mitochondrial function associated with fasting, but not starvation. Our results suggest that the widely adopted liver mitochondrial isolation technique using fasted animals should be reconsidered, and also uncover physiological changes in bioenergetic function associated to nutritional status.

1. Introduction

Overnight fasting is commonly adopted in protocols to obtain isolated liver mitochondria, but the effects of fasting itself on mitochondrial function are poorly characterized. The use of fasted animals is justified by the need to control excess fat and glycogen in homogenates and avoid variations in hormonal signaling. However, the effects of fasting itself on isolated liver mitochondrial functions are, surprisingly, not well characterized.

Fasting and the consequent decrease in blood nutrients lead to decreased insulin levels as well as increased glucagon, thus stimulating glycogenolysis and gluconeogenesis, while suppressing lipogenesis and glycolysis [reviewed in (4)]. At the mitochondrial level, fatty acid oxidation is enhanced [5, 6] due to increased CPT1 expression (the main regulator of mitochondrial fatty acid oxidation [7]), while in the fed state mitochondria contribute toward lipogenesis [4]. However, the effects of overnight fasting on isolated mitochondrial electron transport chain activity and oxidative phosphorylation remain largely unknown. Understanding the effects of fasting on liver mitochondrial function is not only relevant for improved isolated mitochondrial protocols, which very commonly adopt substrates other than fatty acids, but also to gain insight into metabolic adaptations during fasting and liver function control by hormonal action and nutrient availability.

Interestingly, studies testing the effects of glucagon on liver mitochondrial function showed a clear increase in respiration [8–10] and calcium retention capacity [11]. This suggests that fasting may be associated with a boost in mitochondrial function, since glucagon signaling is present. Later studies showed that the increase observed in calcium retention capacity was indirectly related to the classical glucagon signaling pathway, acting through its cellular Ca²⁺ mobilizing effects [12]. Indeed, Ca²⁺ release from the endoplasmic reticulum is also known to increase Ca²⁺ concentrations in the mitochondrial matrix and stimulate oxidative phosphorylation [reviewed in (13)]. Notably, these studies with glucagon administration did not assess the effects of physiological hormonal signaling on mitochondrial function, but rather were conducted by administrating glucagon to fed mice, which promotes simultaneous high levels of insulin and glucagon, as well as high nutrient availability.

In this study, we examined the effects of 15 h (overnight) and short term (4 h) fasting on the function of isolated liver mitochondria. We surprisingly show that both overnight and short-term fasting result in less functional mitochondrial preparations, reducing ADP-stimulated (state 3) and maximal respiration, respiratory control ratios, membrane potentials, and calcium retention capacity. These results suggest that fasting promotes a physiological shift in liver mitochondrial

**Abbreviations:** CRC, calcium retention capacity; ER, endoplasmic reticulum; MCU, mitochondrial calcium uniporter; RCR, respiratory control ratio; CS, citrate synthase.

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bioenergetics toward an overall decrease in oxidative phosphorylation efficiency and capacity, with possible physiological implications for liver energy metabolism.

2. Materials and methods

2.1. Animals and fasting

Male, adult, 8 week old CS78L/6N mice were caged individually and randomly divided into "fasted" or "fed" groups. Mice in the fasted group were moved to new cages 15 or 4 h before the experiments without any access to food, while the mice from the "fed" group were moved to new cages and given chow ad libitum. Under fasting conditions, torpor typically occurs starting at 8 h of fasting [14,15]. All experiments were conducted starting around 10 AM. Animals were kept at a controlled temperature of 22 °C in a 12 h night/day cycle, with lights turning on at 7 AM and going off at 7 PM. Usage of the mice was approved by the local animal care and use committee and followed National Institutes of Health guidelines for humane treatment of animals.

2.2. Mitochondrial isolation

Liver mitochondria were isolated and used for oxygen consumption and calcium uptake experiments employing the same methods described previously by our group [16]. Briefly, fed and fasted animals were euthanized prior to the isolation procedure and had their livers removed, finely minced and homogenized in an isolation buffer containing 250 mM sucrose, 10 mM HEPES and 1 mM EGTA, pH 7.2 (K'). using a glass Potter to obtain liver homogenates. A sample of the homogenates was separated and stored with protease inhibitors at -80 °C for later use. The remaining volume was used to obtain isolated mitochondria by differential centrifugation, with two centrifugation steps at 700g, collecting the supernatant, followed by two centrifugation steps at 10,000g, collecting the pellet containing concentrated isolated mitochondria. After these procedures, the isolated mitochondrial pellet was resuspended in a buffer containing 75 mM mannitol, 25 mM sucrose, 5 mM K2HPO4, 20 mM Tris-HCl, 100 mM KC1 and 0.1% BSA, 1 mM EGTA and 0.5 mM EDTA, pH 7.4 (K'). Experiments were carried out in the same media devoid of EGTA and EDTA, at 37 °C, with 2 μM rotenone and 5 mM succinate as the substrate, which provides better respiratory rates. Similar results to those shown in Fig. 1A and B were observed using malate plus glutamate (1 mM each) as substrates (results not shown). Protein quantification in the samples was performed using the Bradford method.

2.3. Oxygen consumption

Oxygen consumption was measured using a high-resolution Oroboros oxygraph. Mitochondria (250 μg/mL) were suspended in the experimental medium described above. State 3 respiration was measured by adding 1 mM ADP, oligomycin-insensitive respiration by adding 1.5 μg/mL oligomycin and maximal respiration was promoted by successive additions of 0.5 μM CCCP until maximal O2 consumption was observed. Respiratory control ratios were determined as State 3 (ADP/Oligomycin rates).

2.4. Ca2+ uptake assays

Mitochondria (500 μg protein) were incubated in 2 mL experimental buffer containing 0.1 μM Ca2+ Green 5 N and 1 μM MgCl2, Ca2+ Green fluorescence was measured at 506 nm excitation and 532 emission using a F2500 Hitachi fluorimeter at 37 °C with continuous stirring. Ca2+ uptake (50 μM for Fig. 4) were made where indicated in the figures. Using Ca2+ uptake traces, fluorescence values (F) were converted into [Ca2+] using the formula \( [Ca^{2+}] = K_d (F - F_{min})/(F_{max} - F) \). The Kd value was experimentally estimated as the value which corresponds to a 10 μM increase in [Ca2+] after each addition, since the presence of magnesium in the media interferes with the theoretical Kd. Fmax was obtained by adding 100 μL of a saturated Ca2+ solution until no increase in fluorescence was observed, and Fmin was estimated by doing the same with an EDTA solution. Calcium retention capacity was calculated as the total amount of Ca2+ taken up by mitochondria before the onset of mitochondrial permeability transition (nmol Ca2+/mg protein), as seen by the lack of further Ca2+ accumulation and increase in Ca2+ Green fluorescence due to Ca2+ release from mitochondria.

2.5. Mitochondrial membrane potential assay

Membrane potentials were quantified in isolated mitochondria (500 μg/mL) suspended in the same experimental buffer described supplemented with 5 μM safranine O. Uptake was measured by following safranine O fluorescence at 496 nm excitation and 586 nm emission in a F4500 Hitachi fluorimeter. A calibration curve was made for each preparation by incubating mitochondria in a sodium-based potassium-free media with valynomicin, followed by the addition of known KCl concentrations and fitting the resulting membrane potential values (obtained through the Nernst equation) with the observed fluorescence intensity [17].

2.6. Western blots and antibodies

Western blots of mitochondrial samples were carried out by resolving them using SDS-PAGE (20% gels) with 30 μg of protein (quantified by the Bradford method) in each lane, and then transferring the proteins to nitrocellulose membranes. Membranes were stained with Ponceau solution and scanned for later use as a loading control. Primary antibodies and dilutions used were used HSP60 (1:5000; Abcam), TOM20 (1:200, Santa Cruz Biotechnology) and VDAC (1:500, Cell Signaling). Band intensities were quantified using ImageJ and then normalized by the intensities of total protein indicated by Ponceau staining (also quantified using ImageJ).

2.7. Phospholipid quantification

Total phospholipids were extracted from liver homogenates by the method of Bligh & Dyer [18]. Samples (10 μL) were used to quantify phosphorous by the malaquite green method [19]. Phospholipids were analyzed by two-dimensional thin layer chromatography using chloroform/methanol/H2O/NH4OH (aq 30%) (60:37:5:3:1) as the mobile phase for the first dimension and a solution of chloroform/methanol/acetic acid/H2O (170:25:25:6) for the second dimension. Phospholipids were qualitatively visualized by iodine staining, then cardiolipin bands were scraped from silica plates, dissolved in 50 μL chloroform/methanol (1:1) and used for phosphorous quantification by the malaquite green method [19] normalized to protein content.

2.8. Citrate synthase activity

Citrate synthase activity was measured in total liver homogenates and in isolated mitochondria following the increase in 5,5'-dithiobis(2-nitrobenzoic acid) absorbance at 412 nm in the presence of 10 mM acetyl-CoA as described in [20].

2.9. Statistical analysis

Statistical analysis was carried out using two-tailed, unpaired, Student's t-tests. Errors are presented as the standard error of mean (SEM).
3. Results

Overnight fasting reduces mitochondrial oxidative phosphorylation capacity, respiratory control ratios and membrane potentials.

In order to evaluate the effects of overnight fasting on mitochondrial function, mitochondria from the livers of fed (ad libitum food access) and overnight (15 h) fasted mice were isolated and used in oxygen consumption and membrane potential experiments, as described in Materials and Methods. Mitochondria from overnight fasted animals consistently displayed an overall decrease in all measured mitochondrial oxidative phosphorylation parameters (Fig. 1). More specifically, fasting lead to a reduction in both ATP synthesis-linked state 3 and maximal uncoupled respiration (Fig. 1A and B), measured in the presence of added ADP and FCCP, respectively) as well as reduced respiratory control ratios (Fig. 1C), or the relationship between ADP-stimulated respiration and that in the presence of the ATP synthase inhibitor oligomycin. Indeed, a significant decrease in membrane potentials (Fig. 1D and E) was experimentally measured, confirming a loss in oxidative phosphorylation efficiency. Interestingly, although oligomycin-insensitive oxygen consumption was not significantly altered by fasting (suggesting the same absolute amount of proton leak), membrane potentials measured after oligomycin were lower in 15 h fasted mitochondria (Fig. 1E), suggesting similar amounts of proton leak for a lower membrane potential. Since the proton leak is expected to be exponentially increased relative to the membrane potential [21], the presence of similar amounts of proton leak under a condition in which the membrane potential is lower suggests a leakier membrane. Overall, these results clearly demonstrate that fasting promotes a decrease in oxidative phosphorylation efficiency in liver mitochondria.

3.1. Overnight fasting reduces mitochondrial calcium retention capacity

The amount of Ca^{2+} mitochondria are able to accumulate is an indicator of their vulnerability to pathological over-accumulation of this ion [22,23]. Indeed, mitochondrial permeability transition, a non-selective form of inner membrane permeabilization induced by excessive Ca^{2+} and oxidants, participates in many pathological conditions including liver ischemia-reperfusion and acetaminophen-induced damage [16,22-25].

We measured mitochondrial Ca^{2+} accumulation and retention capacity by adding multiple pulses of Ca^{2+} at fixed time intervals and following free Ca^{2+} concentrations in the media using the fluorescent Ca^{2+} probe Calcium Green 5N, which was not taken up by mitochondria (Fig. 2A). The arrows in Fig. 2A indicate Ca^{2+} additions, leading to an immediate increase in medium [Ca^{2+}]; the subsequent downward deflection of the fluorescence curve over time indicates mitochondrial Ca^{2+} uptake, decreasing the fluorescence signal of the membrane-impermeable probe. Ca^{2+} additions were continued until a spontaneous increase in free [Ca^{2+}] in the media was observed (Fig. 2A), promoted by the release of accumulated Ca^{2+} by mitochondria after permeability transition. Our data show a large decrease in calcium retention capacity in mitochondria isolated from the livers of mice fasted overnight (Fig. 2A and B), once again indicating that fasting decreases the overall quality of isolated mitochondrial preparations.

3.2. Liver mitochondrial preparation purity is unchanged by overnight fasting

Since experiments described above were carried out with the same amounts of mitochondrial protein, we questioned if the observed increases in oxygen consumption and calcium retention capacity could be attributed to different contamination of the samples with components.
from other sources (such as microsomes and peroxisomes). To assess this possibility, we performed western blots for three widely accepted marker proteins in our isolated mitochondrial samples: HSP60, TOM20 and VDAC (Fig. 3A and B). None of the markers showed any differences between the samples of the two groups when normalized to total protein, indicating similar mitochondrial purity in the fed and fasted samples. The mitochondrially-specific lipid cardiolipin was also present in equal amounts in mitochondria from the livers of both groups (results not shown), showing that fasting does not lead to a higher abundance of membranes from other sources, which could cause differential contamination. Mitochondria from fasted livers also showed no significant changes in citrate synthase activity, although a tendency toward a decrease was noted (Fig. 3C, p = 0.10). Interestingly, changes in citrate synthase enzymatic content or activity normalized to mitochondrial mass have been observed elsewhere [26,27]. This suggests that the levels of this specific mitochondrial protein are not necessarily constant in the mitochondrial population under different conditions, and brings to light the necessity to use multiple mitochondrial markers as indicators of mitochondrial mass. In total liver homogenates, citrate synthase activity was also not significantly different (Fig. 3D), although the dispersion of data was large, leading us to measure a second mitochondrial marker in the homogenate. Cardiolipin quantities relative to total protein in homogenates were slightly higher in fasted animals (Fig. 3E), suggesting liver mitochondrial mass increases with fasting, which may be a compensatory mechanism for the loss of oxidative phosphorylation capacity observed.

3.3. Short term fasting has similar effects

Our results up to now indicate clearly that, although it does not change the purity of isolated liver mitochondrial samples, fasting promotes changes in mitochondrial function, with a decrease in respiratory capacity and oxidative phosphorylation, as well as higher susceptibility to permeability transition. The fasting protocol used involved the typical overnight fasting almost universally used for isolated liver mitochondrial studies. This, however, leads to the question if this decrease in mitochondrial function is an effect of fasting alone or of torpor, a physiological hypometabolic state which mice often enter after many hours without food [14,15]. Indeed, Brown and Staples [14] have described changes in mitochondrial oxygen consumption and coupling in torpid rodents.

In order to verify if the mitochondrial changes observed were secondary to fasting alone or fasting-induced torpor, oxygen consumption and calcium uptake assays were repeated with a separate group which was fasted for only 4 h, less time than necessary for the induction of the first bout of torpor [14,15], but sufficient time to change hormonal levels. As shown in Fig. 4A, B and C, mitochondria from the 4 h fasted group showed essentially the same changes in oxidative phosphorylation as the 15 h group, with a reduction in ADP-stimulated, maximal uncoupled respiration and respiratory control ratios. Calcium retention capacity was also decreased when compared to the fed group (Fig. 4D and E). These results show that the functional changes observed in mitochondria from fasted mice are not the result of stress responses to poor nutrient availability or torpor-induced metabolic suppression, but rather a physiological change related to the fasted state itself.

4. Discussion

The results presented in this paper show that mice submitted to short or overnight fasting periods undergo a shift in liver mitochondrial bioenergetics characterized by a decrease in maximal respiratory capacity and oxidative phosphorylation. These results are compatible with those of Brown and Staples [14], who found a decrease in oxidative phosphorylation and coupling in fasting-induced torpid mice, and thus suggested this could be a metabolic suppression to save energy in the torpid state. However, we found that only 4 h of fasting, a time which is much lower than needed to induce the first bout of torpor [14,15], was sufficient to promote the same changes, indicating they are induced by fasting itself, not torpor. Indeed, a decrease in mitochondrial respiratory control ratios and membrane potentials such as that observed is not compatible with metabolic suppression, as it could promote more wasteful usage of the available substrates [28].

These results have an immediate practical implication for isolated mitochondrial studies, in which fasting was typically adopted to date, purportedly to avoid excess fat in the liver homogenate. Fasting has also been justified to be useful in mitochondrial isolation to avoid differences in hormonal and nutrient levels that may affect preparation homogeneity. However, we noted no difference in heterogeneity between mitochondrial preparations conducted in fasted versus fed animals to justify this hypothesis. Overall, our data suggest that, for most isolated mitochondrial studies, using fed animals may be not only a better ethical choice, but also one that yields higher quality preparations.

Interestingly, we find that not only electron transport and oxidative phosphorylation are augmented in fed animals, but also the ability to take up and store calcium, limiting Ca2+-induced mitochondrial permeability transition [22]. In this line, fasting has been shown to potentiate liver damage induced by acetaminophen [29], a type of liver damage that is related to the mitochondrial permeability transition [25]. This suggests that the increased resistance to permeability
transition seen in fed animals occurs not only in vitro, but also in vivo, and further supports our isolated mitochondrial results. Of note, decreased Ca^{2+} accumulation capacity is seen in acute short and long-term fasting (as described here), but not in chronic daily caloric intake limitation (such as in caloric restriction), which promotes increased resistance against mitochondrial permeability transition and liver injury [16].

The mechanism in which respiration, membrane potentials and Ca^{2+} uptake are improved in fed versus fasted animals remains to be determined. Based on results in perfused livers, in which glucagon administration in the presence of high nutrient levels promoted increases in mitochondrial respiration and calcium retention capacity [8–11], we suggest that the effects of fasting seen here are not attributable to increased glucagon signaling alone. One contributing factor for the effects of fasting may be nutrient levels and their combined influence with hormonal signaling on the subtypes of mitochondria present. It should also be noted that fasting is a potent inducer of autophagy and mitophagy [30], which, associated with mitochondrial
Fig. 4. Short term fasting promotes similar changes in function to overnight fasting. Experiments showed in Fig. 1 were repeated comparing fed animals to animals fasted for 4 h. (A) Representative oxygen consumption traces. The arrows indicate the addition of ADP, oligomycin and CCCP titrations. (B) Oxygen consumption quantification. (C) Respiratory control ratios. (D) Representative Ca\(^{2+}\) uptake traces in which each arrow indicates a bolus Ca\(^{2+}\) addition. (E) Maximal Ca\(^{2+}\) retention capacity before the onset of permeability transition. * \(p < 0.05\); ***, \(p < 0.001\).

biogenesis, could promote replacement of part of the mitochondrial population with a distinct biochemical profile. A recent paper from Shirihai's group [27] shows that mitochondria associated to lipid droplets in the brown adipose tissue constitute a separate population with a distinct biochemical profile, high coupling and focused on fueling substrates for fatty acid synthesis, while the remaining mitochondria in the brown adipose cells are poorly coupled and associated with fatty acid oxidation [27]. Interestingly, the biochemical characteristics of lipid droplet-associated mitochondrial populations shows similar features to the mitochondria from fed animals in our study, namely higher state 3 and maximal respiration, higher mitochondrial coupling and increased citrate synthase activity when compared to the fastest group [27]. Since fatty acid synthesis (lipogenesis) is upregulated by insulin and normally occurs in the fed state [reviewed in [4]], it is tempting to hypothesize that the liver, similarly to brown adipose tissue, may present more efficient mitochondria when synthesizing lipids in the fed state. Further studies uncovering the mechanisms regulating these shifts would be of interest to understand metabolic fluctuations in liver bioenergetics, both in physiology and pathology.

In brief, our data show that mitochondria isolated from fasted animals show clear differences in their biochemical profile compared to mitochondria from fed animals, which could reflect physiological adaptations to hormonal signaling and nutrient availability. This finding has both implications for understanding bioenergetic regulation in different nutritional states and for standard laboratory mitochondrial isolation protocols, which we suggest should no longer use animals fasted overnight.

Conflict of interest

The authors have no conflict of interest to declare.

Transparency document

The Transparency document associated with this article can be found in online version.

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CHAPTER IV – Impact of mitochondrial dynamics on cellular Ca$^{2+}$ homeostasis

Unpublished data

Preface and author contributions

This chapter contains the results from a side-project based on previous (unpublished) data obtained by my PhD advisor during her visiting researcher grant at the laboratory of Dr. Orian Shirihai (UCLA). Her data showed that mitochondria forced into a pro-fusion phenothype by transfection with a dominant negative form of DRP1 (DRP1-DN) showed higher Ca$^{2+}$ retention capacity (CRC) and uptake rates, while mitochondria forced to a fission phenotype by knockdown of the fusion protein MFN2 showed the opposite, displaying lower CRC and Ca$^{2+}$ uptake rates.

The goal of this side project was to establish if these changes in mitochondrial Ca$^{2+}$ uptake in permeabilized cells would also reflect on physiological modulation of Ca$^{2+}$ signaling in intact cells. In order to assess that, experiments were conducted testing the effects of modulating mitochondrial dynamics on intracellular Ca$^{2+}$ levels, ER Ca$^{2+}$ stores and the process of store-operated Ca$^{2+}$ entry (SOCE), well known to be regulated by mitochondrial Ca$^{2+}$ uptake (as discussed in Chapter I). The results obtained show a clear effect of promoting mitochondrial fission on regulating basal and ER Ca$^{2+}$ levels. Furthermore, SOCE was regulated both by mitochondrial fission and fusion, with higher mitochondrial fusion modulating positively, while more fragmented mitochondria significantly impaired the process. All the data shown in this section are currently being prepared for publication.

Alicia J. Kowaltowski and Sergio L. Menezes-Filho designed the experiments. Alicia J. Kowaltowski conducted the experiments shown in Fig. 1 (with help from Essam
Assali and Nathaniel Miller) and 2, while Sergio L. Menezes Filho conducted the experiments shown in Fig. 3. Ca\textsuperscript{2+} imaging was done using a DMI8 Leica microscope in the laboratory of Dr. Alexandre Bruni-Cardoso, which kindly helped us with the experimental protocol and equipment use.
1. Introduction

Recently, advances in imaging techniques have allowed for detailed studies of mitochondrial morphology and their dynamic changes over time. In these studies, mitochondria were found not only to vary in size and shape in different cell types, but also to rapidly remodel their morphology in response to environmental changes such as nutrient availability (reviewed in Pernas and Scorrano, 2016). Interestingly, these changes in mitochondrial morphology occur not only in response to metabolic cues but also regulate cellular metabolic responses (reviewed in Liesa and Shirihai, 2013). Dynamic changes in mitochondrial morphology additionally regulate organelle turnover, the maintenance of a healthy mitochondrial pool and the interaction between mitochondria and the endoplasmic reticulum (Gottlieb and Shirihai, 2014; reviewed in Pernas and Scorrano, 2016; Klecker, Böckler and Westermann, 2014; Trudeau,).

Many modulators of mitochondrial morphology are well known today. Mitofusin 1 and Mitofusin 2 (MFN1 and MFN2) are outer membrane dynamin-related GTPase proteins that form complexes between neighboring mitochondria and mediate outer membrane fusion. This process is followed by inner membrane fusion mediated by OPA1, a GTPase also involved in cristae remodeling (reviewed in Pernas and Scorrano, 2016). In addition to mediating mitochondrial fusion, MFN2 also plays a key role in Ca\(^{2+}\) signaling by tethering mitochondria to the ER (de Brito and Scorrano, 2008). Increased mitochondrial fusion is associated with enhanced bioenergetic efficiency (reviewed in Liesa and Shirihai, 2013).

Mitochondrial fission, on the other hand, is associated with low bioenergetic efficiency and is controlled by the cytosolic dynamin-related protein 1 (DRP1), that assembles as dimers and oligomers around the fission site. GTP hydrolysis and DRP1
superstructure constriction then promote mitochondrial fragmentation (reviewed in Pernas and Scorrano, 2016). Interestingly, the physical site for mitochondrial constriction seems to be determined by ER-mitochondrial interactions (Friedman et al, 2011).

ER-mitochondrial interactions are important not only for the regulation of mitochondrial morphology, but also for the regulation of intracellular Ca\(^{2+}\) signals (Rizzuto et al, 1998; reviewed in Csordás et al, 2006). Intracellular and intramitochondrial Ca\(^{2+}\), on the other hand, are important metabolic regulators (reviewed in Gunter and Sheu, 2009). While the close interaction between the ER and mitochondria is important for adequate signaling, recent studies have shown that ER-mitochondrial interactions increase excessively in high fat diets, resulting in mitochondrial Ca\(^{2+}\) overload and dysfunction. Disrupting these interactions can increase animal health, despite the diet, demonstrating the importance of mitochondrial Ca\(^{2+}\) in metabolic control (Arruda and Hotamisligil, 2015).

Ca\(^{2+}\) uptake into the mitochondrial matrix is driven by the inner mitochondrial membrane potential, and is mediated by the mitochondrial Ca\(^{2+}\) uniporter (MCU) (Baughman et al, 2011). Although low in affinity relative to the ER, mitochondrial Ca\(^{2+}\) uptake presents high capacity, allowing for the accumulation of large quantities the ion. Within mitochondria, Ca\(^{2+}\) ions act as regulators of important metabolic pathways, determining ATP synthesis rates (Gunther and Sheu, 2009).

Excessive mitochondrial Ca\(^{2+}\) uptake is also disruptive for cell integrity in a number of pathological conditions, including stroke, ischemic heart disease and inflammatory liver conditions (Arruda and Hotamisligil, 2015; reviewed in Duchen, 2000; Murphy and Steenbergen, 2008; Nicholls, 2009). Under these conditions, mitochondrial dysfunction is associated with the mitochondrial permeability transition, a cyclosporin A-
sensitive loss of inner membrane impermeability promoted by Ca\(^{2+}\) overload, oxidative imbalance and protein missfolding (reviewed in Figueira et al, 2013; Biasuto et al, 2016).

We have recently found that mitochondria isolated from animals maintained on a calorically-restricted (CR) diet present increased Ca\(^{2+}\) accumulation capacity and resistance against permeability transition (Amigo et al, 2017). This further supports the notion that dietary interventions may not only affect physiological Ca\(^{2+}\) handling but also modulate damaging effects of supraphysiological Ca\(^{2+}\) accumulation. Interestingly, CR may also modulate mitochondrial morphology, stimulating mitochondrial fusion (Khraiwesh et al, 2014; Cerqueira et al, 2016).

Altogether, a wealth of evidence supports a link between nutritional status, mitochondrial morphology and dynamics, Ca\(^{2+}\) signaling and bioenergetic efficiency. However, a specific and central point that has not been studied yet is if changes in mitochondrial morphology, alone, promote changes in mitochondrial Ca\(^{2+}\) uptake and intracellular Ca\(^{2+}\) signaling.

2. Goals

- Uncover the effects of mitochondrial morphology on mitochondrial Ca\(^{2+}\) handling.
- Test the physiological effects of this modulation on cellular Ca\(^{2+}\) homeostasis.

3. Materials and methods

3.1. Cell culture and adenoviral transfection

C2C12 cells (passage 6-16) were cultured in high glucose DMEM with 10% FBS, 1 mM pyruvate, 100 units/mL penicillin and 1000 μg/mL streptomycin, trypsinized every 2-3 days and typically split 1:7 (Wikstrom et al, 2012). Separately trypsinized plates were
considered separate experiments, and at least 2 passages and 3 repetitions were tested for each experimental condition. Mitochondrial fusion was promoted by infection with an adenovirus containing a dominant negative form of DRP1 (DRP1 DN, from Welgen, Inc.) at a multiplicity of infection (MOI) level of 200. Mitochondrial fission was promoted by MFN2 knockdown using an adenovirus from Welgen at a MOI of 20. Cells were infected when split, and the media was changed after 24 hours. Experiments were conducted 72 hours after infection, when infection rates were maximized (Forni et al, 2016).

3.2. Analysis of mitochondrial morphology

Cells were plated on glass-bottomed plates 72 hours prior to imaging and loaded with Mitotracker Green (200 nM) for 30 min followed by wash-out just prior to the experiment. A Zeiss LSM 880 confocal microscope with Airyscan mode was used for super-resolution imaging, with a 488 nm laser and 63× objective. At least 10 images per experimental condition were collected, the cells were individualized as areas of interest using Image J, and automated mitochondrial circularity and aspect ratios (the proportional relationship between width and height) were measured. Mitochondrial fission is expected to increase circularity and decrease the aspect ratio.

3.3. Ca²⁺ uptake experiments in permeabilized cells

Cells were trypsinized, washed and suspended in 200 µL of 140 mM NaCl, 3 mM KCl, 400 µM KH₂PO₄, 20 mM HEPES, 5 mM NaHPO₃, 5 mM glucose and 1 mM MgCl₂, pH 7 (NaOH). They were counted and kept in this media at room temperature for at most 90 min while experiments were conducted. Ideal cell quantities and digitonin titers were determined by following plasma membrane permeabilization using safranin (see below Kowaltowski et al, 2002) and were found to be 2.5 . 10⁶/mL for C2C12 cells, in the presence of 0.00025% digitonin (added just prior to the trace). Extramitochondrial Ca²⁺
uptake was followed in the permeabilized cells using the florescent calcium probe Ca\textsuperscript{2+} Green (100 nM, Bambrick et al., 2006) in media containing 125 mM KCl, 2 mM KH\textsubscript{2}PO\textsubscript{4}, 10 mM Hepes, 1 mM MgCl\textsubscript{2}, 5 mM succinate, 5 mM malate and 5 mM glutamate, pH 7 (KOH). Fluorescence was measured with constant stirring, in a cuvette fluorimeter at 506 nm excitation and 532 nm emission. Where indicated in the figures, successive additions of 50 µM CaCl\textsubscript{2} were made, until Ca\textsuperscript{2+} accumulation capacity was exhausted, as indicated by a lack of further uptake and/or Ca\textsuperscript{2+} release. A calibration curve was constructed using known CaCl\textsubscript{2} concentrations, and maximal calcium uptake capacity and initial uptake rates were calculated for each trace. Where indicated, 1 µM cyclosporin A (CsA), a permeability transition inhibitor, or 1 µM ruthenium red (RR), an MCU inhibitor, were present.

3.4. Ca\textsuperscript{2+} imaging with Fura2 in intact cells

Three days prior to the experiment, C2C12 cells were plated in 4-compartment glass bottom cell culture dishes from Greiner (No 627871) and were then separated in three groups: one infected with the DRP1-DN (DRP1-DN group) adenovirus, another with the MFN2-KD adenovirus (MFN-KD group) and the last with no transfection (control group), following the same transfection protocol described in the previous sections. Fura2-AM loading and imaging protocols were adapted from (Arruda et al, 2017), with modifications. On the day of the experiment, the culture media was removed and cells were washed twice with the experimental buffer containing 10 mM HEPES, 150 mM NaCl, 4 mM KCl, 1 mM MgCl\textsubscript{2} and 10 mM D-Glucose (pH 7.4) and then incubated with 10 µM Fura2-AM and 0.1% pluronic acid and 2 mM CaCl\textsubscript{2} in that same media for 40 minutes at 37°C and 5% CO\textsubscript{2}. After the incubation, the media was removed and cells were again washed twice in the experimental buffer. After that, cells were incubated in 1
mL of the buffer and then placed inside the chamber of a Leica DMI-8 microscope equipped with a Fura2 filter (Leica Microsystems). Fura2 cytosolic Ca$^{2+}$ imaging was conducted by alternatively illuminating the cells with wavelengths of 340 and 387 nm respectively, with images being collected every 5 seconds. Cytosolic Ca$^{2+}$ levels are shown in the figures as the 340/387 ratios for the Fura2 channels. Intensities were calculated using the FIJI Image J extension by converting the videos obtained in each channel to grayscale and then plotting the mean gray values over time for each cell. At the mark of 30 s after the beginning of the experiment, 2 µM thapsigargin was added to the experimental media in order to promote ER Ca$^{2+}$ release, and at the mark of 630 s, 2 mM CaCl$_2$ was added to the experiment in order to trigger Ca$^{2+}$ entry through SOCE. Experiments were conducted in three separate days, with 7-10 cells being analyzed for each day. Cytosolic Ca$^{2+}$ (340/387 nm) increase rates after the addition of 2 mM CaCl$_2$ (Fig 2C) were measured by adjusting a linear fit using Origin 8 Pro software.

3.5 Statistical analysis

Comparisons were made using Graphpad Prism software, with t-tests for simple comparisons between two groups and ANOVA for multiple comparisons.

4. Results

4.1 Mitochondrial dynamics regulate mitochondrial Ca$^{2+}$ handling in permeabilized C2C12 myoblasts

Fig. 1 shows typical images (Fig. 1A) and quantifications of circularity and aspect ratios (Fig. 1B and C) of Mitotracker Green-stained mitochondria in C2C12 cells under control conditions and when infected with DRP1 DN or MFN2 KD adenoviruses. DRP1
disruption results in a decrease in mitochondrial circularity and increase in aspect ratio typical of enhanced mitochondrial fusion, while MFN2 knockdown promotes more circular mitochondria with lower aspect ratios typical of enhanced mitochondrial fission. Neither treatment affected the area occupied by mitochondria (Fig. 1D), possibly because of the large heterogeneity of mitochondrial mass in these cell lines. Overall, this experiment demonstrates that we were able to modulate mitochondrial morphology in the two cell lines used, without compromising cell viability.

Figure 1: Mitochondrial morphology in C2C12 cells is modulated by DRP1 and MFN2. C2C12 cell (as indicated) mitochondria were marked with MitoTracker Green and imaged as described in the Methods section. Mitochondrial areas relative to the cell, circularity and aspect ratios were quantified from these images. N > 10, *, p < 0.05; ** p < 0.01; *** p < 0.001; relative to wild-type cells.
Next, we verified if the changes in mitochondrial morphology affected Ca\(^{2+}\) uptake by these organelles using permeabilized cell preparations (see Methods). Results for these experiments are shown in Fig. 2, with DRP1 DN cells showing highly increased maximal Ca\(^{2+}\) retention and Ca\(^{2+}\) uptake rates as well, while MFN2 KO cells showed the opposite. Ca\(^{2+}\) uptake measured under these conditions was attributable to mitochondria, since complete inhibition was observed in the presence of the MCU inhibitor ruthenium red (RR). These results show a clear role for mitochondrial dynamics in regulating mitochondrial Ca\(^{2+}\) handling in these cells, with higher levels of mitochondrial fusion being associated with higher mitochondrial Ca\(^{2+}\) retention and faster Ca\(^{2+}\) uptake.
Figure 2: Ca\textsuperscript{2+} uptake in C2C12 cells is modulated by mitochondrial morphology.
Ca\textsuperscript{2+} uptake was measured in permeabilized cells as described in the Methods section, in control cells or cells infected with DRP1 DN or MFN2 KD viruses, as indicated. Where indicated, CsA and RR were present. The upper panels show typical traces in which Ca\textsuperscript{2+} additions are marked by arrows. (A) Control Ca\textsuperscript{2+} uptake traces with RR (an MCU pharmagical blocker) and CsA (a negative modulator of mPT which enhances CRC), note the complete absence of Ca\textsuperscript{2+} uptake with RR and the increased CRC with CsA. (B) Representative trace of the Ca\textsuperscript{2+} uptake experiments in control, DRP1 DN and MFN2 KO cells, which are quantified in the lower panels. (C) Quantification of mitochondrial CRC in permeabilized C2C12 cells. (B) Quantification of Ca\textsuperscript{2+} uptake rates in the same experiments. N ≥ 5. *, significantly different from the non-infected cells under the same conditions. #, significantly different from cells in the absence of CsA or RR. N ≥ 5. *, significantly different from the non-infected cells under the same conditions. #, significantly different from cells in the absence of CsA or RR.

4.2 Mitochondrial dynamics regulate cellular Ca\textsuperscript{2+} homeostasis

The previous experiments were conducted in permeabilized cells, a condition in which the components of the cytoplasm are vastly diluted in the experimental media,
while also exposing mitochondria to raises of tenths of micromolar of \(\text{Ca}^{2+}\) in single bolus additions. We sought next to answer if the changes observed in permeabilized cell mitochondrial \(\text{Ca}^{2+}\) uptake had an impact on intact cell \(\text{Ca}^{2+}\) signalling. In order to assess that possibility, we decided to perform \(\text{Ca}^{2+}\) measurements in intact C2C12 cells by loading them with Fura2, a ratiometric cytosolic \(\text{Ca}^{2+}\) probe which allows for live imaging of \(\text{Ca}^{2+}\) in intact cells. After the Fura2 loading, \(\text{Ca}^{2+}\) levels in the cells were monitored in real time in a Leica DMI8 microscope (as described in Methods), where cytosolic \([\text{Ca}^{2+}]_c\) was followed sequentially under three different conditions: basal \(\text{Ca}^{2+}\) levels, thapsigargarging-induced \(\text{Ca}^{2+}\) release from the ER (which was measured until normalization of basal \(\text{Ca}^{2+}\) levels) and finally the increase in cytosolic \(\text{Ca}^{2+}\) after triggering store-operated \(\text{Ca}^{2+}\) entry (SOCE). As shown in Fig. 3B, induction of mitochondrial fragmentation by MFN2 KD altered all of these parameters, promoting lower basal \(\text{Ca}^{2+}\) levels (both before and after ER depletion with thapsigargin), lower thapsigargin-induced ER \(\text{Ca}^{2+}\) release as well as lower peak cytosolic \(\text{Ca}^{2+}\) levels after SOCE. Additionally, \(\text{Ca}^{2+}\) entry rates during SOCE were slower in MFN2 KD cells (Fig. 3C). Conversely, induction of mitochondrial fusion with DRP1 DN did not impact basal \(\text{Ca}^{2+}\) levels, ER \(\text{Ca}^{2+}\) release or final SOCE \([\text{Ca}^{2+}]_c\) levels, but did produce a statistically significant increase in the \(\text{Ca}^{2+}\) entry rates (Fig. 3C).

These results collectively suggest that mitochondrial dynamics control several aspects of \(\text{Ca}^{2+}\) homeostasis, at least in C2C12 myoblasts. Higher mitochondrial fragmentation correlates with lower cytosolic \(\text{Ca}^{2+}\) levels, lowered ER \(\text{Ca}^{2+}\) content and impaired SOCE, results in line with previous findings that the process of mitochondrial \(\text{Ca}^{2+}\) uptake positively regulates SOCE (Samanta, Douglas and Parekh, 2014). While it is easy to associate the reduced levels of ER \(\text{Ca}^{2+}\) release with impaired \(\text{Ca}^{2+}\)
replenishing in the organelle through SOCE, a phenotype commonly observed in cells made defective in the process through knockdown of protein components of the SOCE machinery like STIM1 (Arruda et al, 2017; Darbellay et al, 2009), the changes in basal Ca^{2+} levels seem harder to explain, and could be attributed to different modulations of the Ca^{2+} homeostasis machinery by mitochondria. It is interesting to note, however, that another study conducted using human primary myoblasts with STIM1 knockdown found a very similar phenotype where the STIM1 KD myoblasts presented lowered basal cytosolic [Ca^{2+}] concentrations, suggesting that store-operated channels could play a role in regulating basal cytosolic Ca^{2+} in myoblasts (Darbellay et al, 2009).
Figure 3: Mitochondrial dynamics control store-operated Ca\(^{2+}\) entry in C2C12 Myoblasts. (A) Representative experimental trace of the quantified Fura2 ratios corresponding to cytosolic [Ca\(^{2+}\)]. The first arrow corresponds to the addition of thapsigargin at 30 s, the second arrow corresponds to the addition of 2 mM CaCl\(_2\) at 630 s. (B) Average peak Ca\(^{2+}\) levels during basal (prior to thapsigargin addition), thapsigargin (corresponding to the thapsigargin-induced Ca\(^{2+}\) peak), basal 2 (after normalization of Ca\(^{2+}\) levels post thapsigargin, but prior to the 2 mM CaCl\(_2\) addition) and SOCE (after the maximal [Ca\(^{2+}\)] is achieved after the addition of 2 mM CaCl\(_2\)). (C) Cytosolic Ca\(^{2+}\) increase rates after Ca\(^{2+}\) addition in control, DRP1 DN and MFN2 KD C2C12 myoblasts. *, significantly different from the non-infected (control) cells under the same conditions. #, significantly different from DRP1 DN cells transfected (for the MFN2 KD cells).
5. Discussion

Our results uncover interesting new mechanisms of the modulation of Ca\(^{2+}\) homeostasis and signaling by mitochondria, namely the modulation of mitochondrial Ca\(^{2+}\) handling, cytosolic Ca\(^{2+}\) levels, ER Ca\(^{2+}\) content and SOCE activation by mitochondrial dynamics. Given that mitochondrial Ca\(^{2+}\) handling itself has been shown to be a direct modulator of several aspects of Ca\(^{2+}\) signaling, including the modulation of Ca\(^{2+}\) release by ER Ca\(^{2+}\) channels and SOCE (Hajnóczky and Thomas, 1999; Samanta, Douglas and Parekh, 2014; reviewed in Bagur and Hajnóczky, 2017), the observed changes in mitochondrial Ca\(^{2+}\) handling shown in permeabilized cells are probably directly responsible for the effects described in intact cells. Since mitochondrial dynamics have been shown to be modulated in several distinct cellular contexts, including changes in nutrient availability and progression through the cell cycle (reviewed in Liesa and Shirihai, 2013; Horbay and Bilyy, 2016), the presence of a mechanism by which mitochondrial Ca\(^{2+}\) homeostasis and cellular Ca\(^{2+}\) signaling is modulated by mitochondrial dynamics is highly relevant to the regulation of Ca\(^{2+}\) signaling in these contexts.

In brief, our work shows for the first time a role for the shape of the mitochondrial network in regulating processes of mitochondrial Ca\(^{2+}\) uptake and cellular Ca\(^{2+}\) homeostasis, consequently also revealing a novel physiologically relevant situation in which mitochondrial Ca\(^{2+}\) handling can be modulated and fine-tuned by the cell. Further studies will be needed to clarify the relevance of these processes in other cell types besides myoblasts and also to evaluate their possible interference in the large variety of Ca\(^{2+}\) signals present in different contexts and cell types.
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Chapter V – General discussion and conclusions

1. General discussion

Mitochondria have long been known to be central players in metabolism due to their role in energy homeostasis through ATP production in oxidative phosphorylation. Given that role, it is not surprising that changes in the nutritional status would impact mitochondrial function in order to fine-tune metabolism and optimize its processes according to the availability of substrates (reviewed in Guarente, 2008). Ca\(^{2+}\) signaling had also been shown to impact mitochondrial function, both directly through increases in [Ca\(^{2+}\)] in the mitochondrial matrix and indirectly by promoting mitochondrial dynamics through the induction of DRP1 phosphorylation and controlling mitochondrial motility in the cell (Jeyaraju, Cisbani and Pellegrini, 2009; reviewed in Yi, Weaver and Hajnóckzky, 2004). However, the way in which the nutritional status could impact mitochondrial Ca\(^{2+}\) handling, allowing for the mitochondria themselves to interfere with pathological conditions of Ca\(^{2+}\) overload and physiological conditions of Ca\(^{2+}\) signaling, had not been addressed in the literature until our first publication in 2017 showing the impact of CR on brain mitochondrial Ca\(^{2+}\) handling (Amigo et al, 2017). The results shown here not only expand the previous results, showing that the impact of CR on mitochondrial Ca\(^{2+}\) handling also occurs in other tissues, through different mechanisms (Chapter II). We also clarify the impact of fasting on liver mitochondrial function both regarding mitochondrial Ca\(^{2+}\) handling and oxidative phosphorylation (Chapter III), which was surprisingly not properly characterized until now.

To that end, the effects of 4 months of CR were tested on mouse liver mitochondria. Our results shown here in Chapter III (Menezes-Filho et al, 2017)
demonstrate that CR does in fact modulate mitochondrial Ca\textsuperscript{2+} handling in the liver as well, promoting an increased CRC and faster Ca\textsuperscript{2+} uptake rates. This leads to protection against ischemia/reperfusion injury, a condition in which cellular Ca\textsuperscript{2+} overload and mPT occur (reviewed in Rohrbach et al, 2014; Lempiäinen et al, 2013; Ran et al, 2015). Curiously enough, these results could not be completely attributed to CyPD like in the brain, but rather to the negative modulation of mPTP by ATP (Chapter II).

Regarding the modulation of liver mitochondrial function by fasting, previous work studied the effects of glucagon signalling on both mitochondrial respiration and CRC, all of which were increased by the intraperitoneal administration of the hormone (Halestrap, 1987; reviewed in Jensen et al, 1983; LaNoue, Strzelecki and Finch, 1984; Taylor et al, 1980). This seemed to suggest that the effects of fasting on liver mitochondria were already described and were related to an overall increase in mitochondrial function. Results from our lab, however, suggest that livers from animals undergoing the typical 16 h overnight fasting actually yielded preparations of mitochondria with lower oxidative phosphorylation capacity, which led us to design a study to test the effects of fasting in liver mitochondrial function (Chapter III). At first, we sought to test if the differences in mitochondrial function from the samples obtained could be due to differences in purity of the mitochondrial samples obtained by differential centrifugation or to fasting-induced torpor, which had already been shown to suppress oxidative phosphorylation and lower mitochondrial coupling in the liver of rodents (Brown and Staples, 2010). Our results, however, show that mitochondrial purity was unchanged and that reduced mitochondrial function persisted under conditions of fasting which did not induce torpor. This suggests that fasting does actually decrease mitochondrial oxidative phosphorylation capacity, coupling and CRC, the opposite of what was believed to be the case given the studies.
with glucagon (Chapter III). One possible explanation for these contradictory results is
the fact that such studies were conducting by administrating glucagon to fed animals,
promoting the presence of both high glucagon, insulin and nutrients, which is very
different from what actually occurs in fasting. Also, both the increase in CRC and in
oxidative phosphorylation can be attributed to the Ca\(^{2+}\)-mobilizing actions of glucagon
(reviewed in Denton, 2009; Amigo et al, 2013), possibly explaining this apparent
contradiction.

Finally, in Chapter IV we show unpublished results (currently in preparation as a
manuscript for publication) which reveal a novel mechanism of Ca\(^{2+}\) signalling
modulation by mitochondrial dynamics, which is a process which is itself regulated by
the nutritional status (Liesa and Shirihai, 2013), possibly establishing a novel link
between nutritional status and the regulation of Ca\(^{2+}\) signaling.

2. General conclusion

In this work we were able to expand our results regarding effects of CR on
mitochondrial Ca\(^{2+}\) handling, showing them not to be restricted only to the brain, but also
occuring in the liver, while also establishing a role for CR in the protection against
pathological conditions of Ca\(^{2+}\) overload by the modulation of mitochondrial Ca\(^{2+}\)
handling. We also clarified the physiological effects of fasting on mitochondrial function,
including Ca\(^{2+}\) handling and oxidative phosphorylation. Finally, we were able to propose
a new mechanism for the regulation of Ca\(^{2+}\) signaling by mitochondrial dynamics, which
could reveal a link between the modulation of mitochondrial dynamics by nutrient
availability and shaping of Ca\(^{2+}\) signaling. Overall, we uncovered effects of nutritional
status on mitochondrial Ca\(^{2+}\) handling, both in pathology and physiology, which
contributes toward our understanding and opens up new exciting possibilities for further discovering new mechanisms of the regulation of mitochondrial function and metabolism in general.
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