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Beyond the TCA cycle: new insights into mitochondrial calcium regulation of oxidative phosphorylation

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Abstract

Mitochondria oxidative phosphorylation is broadly regulated, and mitochondrial Ca^{2+} is increasingly recognized to impact substrate flux under both physiological and pathological conditions. Under physiologic conditions, mitochondrial Ca^{2+} enters through the mitochondrial Ca^{2+} uniporter and boosts ATP production. However, maintaining Ca^{2+} homeostasis is crucial as too little Ca^{2+} inhibits adaptation to stress, while Ca^{2+} overload can trigger cell death. In this review, we discuss new insights obtained over the past several years expanding the relationship between mitochondrial Ca^{2+} and oxidative phosphorylation, with most data obtained from heart, liver, or skeletal muscle. Two new themes are emerging. First, beyond boosting ATP synthesis, Ca^{2+} appears to be a critical determinant of fuel substrate choice between glucose and fatty acids. Second, Ca^{2+} exerts local effects on the electron transport chain indirectly, not via traditional allosteric mechanisms. These depend critically on the transporters involved, such as the uniporter or the Na^{+}-Ca^{2+} exchanger. Alteration of these new relationships during disease can be either compensatory or harmful and suggest that targeting mitochondrial Ca^{2+} may be of therapeutic benefit during diseases featuring impairments in oxidative phosphorylation.

1. Introduction

Ca^{2+} is a ubiquitous divalent ion essential for cellular excitability, mechanical contraction, signaling, and metabolism(1). Studies over several decades have clearly revealed that Ca^{2+} within the mitochondria is a potent stimulator of NADH production, via its allosteric potentiation of the activity of membrane transporters, tricarboxylic acid (TCA) cycle dehydrogenases, and pyruvate dehydrogenase phosphatase. The net result of such regulation is a two-fold increase in ATP production. These well-established results have been reviewed previously (2–5). The goal of this mini-review is to discuss the most recent discoveries regarding mitochondrial Ca^{2+} signaling in relation to the electron transport chain (ETC) and oxidative phosphorylation (OXPHOS) in health and disease. These more recent investigations have expanded the mechanism of Ca^{2+} regulation beyond direct binding and

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allosteric effects on mitochondrial enzymes, revealing a multitude of indirect mechanisms that alter the stability, function, and fluxes through OXPHOS (Fig. 1). In many cases, these novel forms of regulation depend on local regulation between the Ca^{2+} transporters and energetic complexes involved, recapitulating the theme that Ca^{2+} signaling is often a very local phenomenon.

2. Ca^{2+} import into the mitochondria

Ca^{2+} is normally sequestered in the endoplasmic reticulum (ER) to maintain a low cytoplasmic concentration of ~100 nM (6, 7). In the ER, Ca^{2+} levels can range from 200 to 500 μM while intra-mitochondrial free Ca^{2+} levels are typically <100 μM (8, 9). The difference in concentrations within various organelles allow for transient cytoplasmic increases by several orders of magnitude (1–3μM), allowing the cell to utilize Ca^{2+} as a signaling molecule.

The various Ca^{2+} transporters involved in mitochondrial Ca^{2+} uptake have been well defined and reviewed previously(10, 11). Briefly, Ca^{2+} ions cross the outer mitochondrial membrane (OMM) through voltage-dependent anion channels, then the inner mitochondrial membrane (IMM) primarily through the mitochondrial calcium uniporter (12–15). This Ca^{2+} channel complex is made up of a pore-forming subunit (MCU), gate-keeping subunits (MICU1–3), an inhibitory subunit (MCUb), and a bridging accessory subunit (EMRE)(15–19). It also associates frequently with other proteins that help regulate Ca^{2+} influx. To avoid confusion, we refer to the channel complex from here onwards as the uniporter and use subunit abbreviations (e.g. MCU) to refer to specific components of the channel. Through the use of voltage-clamp electrophysiology to isolate mitoplasts (mitochondria devoid of their outer membrane), early studies showed that despite the sea of various cations, the uniporter is highly selective for Ca^{2+} (20). This is an extreme structural specialization to prevent futile depolarization of the inner mitochondrial membrane potential ($\Delta\Psi$) by influx through the uniporter of highly-abundant cytoplasmic ions such as K^+, which is ~6 orders of magnitude more abundant than Ca^{2+} in the cytoplasm. At resting cytosolic Ca^{2+} levels, MCU activity is kept low by the gate-keeping MICU1–3 subunits which block the pore, while at higher Ca^{2+} levels, structural rearrangements allow Ca^{2+} access to the pore and lead to rapid Ca^{2+} influx into the mitochondrial matrix (16). Other mitochondrial influx pathways have been occasionally reported, but their abundance and relevance to OXPHOS have not been well defined(11).

3. Export of mitochondrial calcium through NCLX, LETM1, TMBIM5, and PTP

To prevent Ca^{2+} overload and to maintain homeostasis, mitochondria utilize several Ca^{2+} extrusion strategies. Mitochondrial Ca^{2+} export is mediated by a Na^+-Ca^{2+} exchanger (NCLX, encoded by the *SLC8B1* gene), which couples Ca^{2+} efflux from the matrix to Na^+ influx (21, 22), and Ca^{2+}-H^+ exchangers (encoded by LETM1 and TMBIM5, though some controversy exists regarding these) (23–26). NCLX is essential for maintaining mitochondrial homeostasis. Without NCLX, toxic Ca^{2+} overload leads to organismal lethality (27). Similarly, LETM1 mutations lead to Wolf-Hirschhorn Syndrome, a rare

developmental disorder characterized by intellectual disability, seizures, and craniofacial abnormalities (28). Unlike NCLX, the exact mechanism of LETM1 exchange is somewhat controversial. Although studies across multiple labs have clearly established that LETM1 alters Ca^{2+} efflux (24, 29–31), other research suggests it may be involved in K^+-H^+ exchange (32). TMBIM5 is a newly discovered potential Ca^{2+}-H^+ exchanger, with one possibility being that LETM1 and TMBIM5 are both subunits of a larger Ca^{2+}-H^+ exchanger complex.

4. Overview of classical Ca^{2+} allosteric regulation of the TCA cycle

In classic studies of mitochondrial enzymology, Ca^{2+} was found to be a key allosteric regulator of multiple mitochondrial enzymes of the TCA cycle, increasing production of NADH and ultimately leading to an approximately two-fold enhancement of ATP synthesis (2, 33–36). Such increases are most evident in cardiac or skeletal muscle during periods of stress or exercise, where increases in cytoplasmic Ca^{2+} during β-adrenergic stimulation lead to increases in matrix Ca^{2+}. Such a mechanism matches ATP production to energetic demands during stress. In particular, Ca^{2+} activates several matrix dehydrogenases: isocitrate dehydrogenase (IDH) (37, 38), α-ketoglutarate dehydrogenase (AKGDH) (39, 40), and pyruvate dehydrogenase (PDH) (41). Original descriptions state a $K_{0.5}$ of IDH at [Ca^{2+}] of 5–50μM (42, 43) while the K_m of AKGDH is 0.2μM with ADP or ~2μM with ATP (43). An alternative route of electrons entering the ETC is through the glycerol-3-phosphate shuttle (G3PS). Dihydroxyacetone is converted into glycerol-3-phosphate (G3P) by cystolic glycerol-3-phosphate dehydrogenase (GPD). Mitochondrial GPD2 oxidizes G3P when it diffuses into the intermembrane space. These electrons are transferred to coenzyme Q, providing electrons for OXPHOS. It has been reported that GPD2 is activated by Ca^{2+}-binding (44–46).

Several IMM-embedded metabolite carriers have Ca^{2+} binding EF-hands facing the intermembrane space, which is similar to the cytoplasm in its Ca^{2+} levels. Ca^{2+} binding to these carriers enhances their activity (47–49). Such calcium-binding mitochondrial carriers (CaMC) can be divided into two groups, the aspartate/glutamate exchangers (AGCs, aralar and citrin) or short CaMCs (SCaMC) such as ATP-Mg^{2+}/P_i exchangers. AGCs can be activated by relatively low Ca^{2+} levels, similar to resting cytosolic levels, whereas SCaMCs can require higher (micromolar) levels of Ca^{2+} and import ADP into the mitochondria. Deficiency in SCaMC-3/Slc25a23 results in blunted respiration, decreases in ATP, and Ca^{2+} deregulation(50, 51).

Inside the matrix, the imported aspartate or glutamate can be converted to α-ketoglutarate, the substrate needed for AKGDH, or increase citrate and subsequently isocitrate, used by IDH. Ultimately, enhancing TCA dehydrogenase activity boosts NADH production, feeding the ETC at Complex I and ultimately enhancing ATP production (52). In support of this model, deletion of the Ca^{2+} uniporter limits the ability of organisms to exercise or increase heart rate during periods of stress.

Although this has been the traditional model, recent analyses across mitochondria, cells, and organs, suggests that allosteric effects of Ca^{2+} on the TCA cycle may be less

prominent than previously thought. In these studies, oxygen consumption, ATP production, or organ function (e.g. muscle contraction) in several cases has been preserved even when components of the uniporter are deleted (53–58). Although Ca^{2+} is still present in the matrix even after MCU deletion, these data suggest that lack of Ca^{2+}-induced TCA cycle acceleration can perhaps be compensated by other mechanisms. The newer emerging paradigm suggests that though Ca^{2+} can clearly affect TCA flux and ATP production, its primary effect may occur upstream, in helping determine the efficiency of fuel substrates entering the TCA cycle.

5. Inhibition of the uniporter alters Ca^{2+} regulation of pyruvate flux, leading to a switch in substrate preference towards fatty acids for OXPHOS.

Recent studies examining cardiac- and skeletal muscle-specific knockouts of MCU, the pore-forming uniporter subunit, reveal that Ca^{2+} may also be a primary regulator of mitochondrial fuel substrate preference (59–61). This occurs by Ca^{2+} regulation of the conversion of pyruvate to acetyl-CoA. Pyruvate dehydrogenase (PDH) is inhibited by phosphorylation by PDH kinases, while PDH phosphatases enhance its activity. By enhancing the activity of pyruvate dehydrogenase (PDH) phosphatase, Ca^{2+} indirectly boosts PDH. Because pyruvate conversion to acetyl CoA is downstream of glycolysis, lowering Ca^{2+} levels inhibit this pathway. Therefore, in these several models, there is shift of substrate utilization away from glucose towards fatty acids. In particular, after deletion of MCU, investigators found increased rates of fatty acid relative to glucose oxidation, increased PDH phosphorylation, lower levels of malonyl CoA, a key inhibitor of fatty acid oxidation (via CPT1), and activated forms of enzymes involved in β-oxidation, including malonyl CoA decarboxylase and β-hydroxyacyl CoA dehydrogenase. Taken together, these results suggest that, by altering PDH activation, lower levels of Ca^{2+} produce a profound metabolic rewiring of substrate utilization. Intriguingly, in a separate study, altering pyruvate metabolism also produced a reciprocal change in uniporter activity (62). In several human and mouse cell types, inhibiting glycolysis or mitochondrial pyruvate import led to increased expression of the uniporter gatekeeping subunit MICU1, which apparently inhibited mitochondrial Ca^{2+} uptake and reduced matrix Ca^{2+} levels. The authors posited this mechanism may prevent toxic mitochondrial Ca^{2+} overload during bioenergetic crises caused by nutrient stress.

6. Ca^{2+} regulation of the ETC and F_1-F_O-ATP synthase under normal physiology

The data on Ca^{2+} regulation of flux rates through the ETC and ATP synthase has been somewhat controversial. Multiple early studies on isolated mitochondria or enzyme systems showed Ca^{2+}-dependent boosting of ATP production rates and flux through the ETC (2). However, allosteric binding sites for Ca^{2+} at physiological concentrations in these systems have been difficult to establish, though there may be Ca^{2+} induced changes under pathophysiological conditions. Two recent studies of cardiac and skeletal mitochondria using detailed measurements of several intermediate steps in OXPHOS failed to find evidence for Ca^{2+}-induced enhancement of flux through the ETC or ATP synthase (52, 63). In

these cases, the Ca^{2+}-induced boost in ATP production was explained by its effects on substrate transport, TCA cycle flux, and changes in mitochondrial membrane potential ($\Delta\Psi$), rather than direct effects on the ETC or ATP synthase. In contrast, in another study using skeletal muscle mitochondria, the authors found >2-fold Ca^{2+}-induced increases in conductances through the ETC (distributed across Complexes I, III, and IV) and the ATP synthase (36). Of note, these authors isolated the effects on the ETC by normalizing to NADH production (accounting for effects due to substrate transport or TCA flux); and the effects on the ATP synthase by normalizing to $\Delta\Psi$ (accounting for effects on the ETC). Explanations for these discrepancies remain open questions, with possibilities including the assays (e.g. respirometry, fluorescent reporters, cytochrome redox absorbance) and the usage of a creatine kinase clamp to mimic physiological constraints, as Ca^{2+} stimulation of respiration is minimized under the typical assays using saturating levels of ADP (64). Nevertheless, despite the somewhat murky status of direct allosteric regulation of the ETC and ATP synthase by Ca^{2+}, there is a growing body of evidence suggesting mitochondrial Ca^{2+} is an important indirect regulator of these complexes. We review recent data for these below.

6.1. Complex I

NADH is the substrate utilized by Complex I of the electron transport chain (ETC). Complex I is the first and largest complex of the ETC, consisting of 44-subunit. This L-shaped complex consists of two domains, a transmembrane domain responsible for pumping H^+ into the intermembrane space and a matrix arm that protrudes into the matrix space. The matrix arm oxidizes NADH and transfers the electron (e^-) down its iron-sulfur (Fe-S) clusters until it reaches coenzyme-Q (CoQ). CoQ shuttles electrons to Complex III (65–67). Current evidence suggests that Ca^{2+} indirectly stimulates Complex I through increasing the ratio of NADH/NAD^+ mostly through the matrix dehydrogenases involved in the TCA.

To date there is no evidence suggesting Ca^{2+} binding to Complex I. Instead, our group recently discovered a Complex I-uniporter interaction, through which the uniporter appears to exert a protective effect on Complex I, a phenomenon we termed Complex I Induced Protein Turnover (CLIPT) (Fig. 2A) (68). In normal physiologic conditions, some electrons leak out during transfer from NADH to CoQ at a low rate (~1%), producing potentially toxic reactive oxygen species (ROS) that may locally harm Complex I. We found that the N-terminal domain of MCU interacts with Complex I near the site of electron transport through the Fe-S clusters. Leaking electrons tend to oxidize MCU, leading to its degradation. This constant MCU turnover maintains a basal low rate of uniporter channels, potentially protecting Complex I itself from damage caused by this electron leak. In fact, loss of uniporter components has been associated with a near stoichiometric loss of Complex I in cardiac muscle (59, 69, 70).

6.2. Complex II

Complex II, also known as succinate reductase, is unique in that it is the only one that does not pump protons across the IMM and is directly involved in both the TCA and ETC (71). It contains four protein subunits. It is the second point of electron entry. Complex II couples succinate oxidation to FAD reduction in the TCA cycle, and subsequent $FADH_2$ oxidation to

CoQ reduction in the ETC. There is no clear evidence of Ca^{2+} regulation of Complex II to date.

6.3. Complex III

Complex III, also known as cytochrome c oxidoreductase, is a dimeric structure (72). Electrons from both Complex I and II arrive at Complex III in the form of ubiquinol ($CoQH_2$). At Complex III, a recycling electron loop called the Q-cycle occurs at the two CoQ binding sites. Electrons are ultimately shuttled over to Complex IV by cytochrome c, while $4H^+$ from 2 $CoQH_2$ are released into the intermembrane space. Although limited older data using Complex III-specific substrates suggested Ca^{2+} enhancement of flux (73), no specific binding sites or direct allosteric regulation has been confirmed under physiological conditions. Nevertheless, an elegant recent study revealed indirect effects of Ca^{2+} on Complex III function under pathophysiological conditions (see section 7.2 below).

6.4. Complex IV

The final electron destination is at Complex IV, cytochrome c oxidase, a 13-subunit structure containing 4 redox-active metal centers (74). Two A-type hemes and two copper centers allow electron transfer from cytochrome c to oxygen, the final electron acceptor. Such transfer is coupled to a pumping of 4 H^+ to the intermembrane space. Complex IV has a Ca^{2+} binding site sensitive to Na^+ and Ca^{2+}, facing the intermembrane space (75). Although normally bound to Na^+ at low cytosolic Ca^{2+} levels, high Ca^{2+} ~1μM (previously reported to be 20–30μM (76, 77)) displaces the Na^+ and inhibits activity with an IC_{50} ~0.5μM, though this effect may be evident only during very slow Complex IV activity and may have minimal effect under more rapid physiological rates. This may be an endogenous way to slow down respiration at high Ca^{2+} levels if substrates are limited (75).

Beyond directly binding Ca^{2+}, Complex IV also may have an interaction with the uniporter. A transmembrane protein of unclear function, MCU regulator 1 (MCUR1), has been identified as both a Complex IV assembly factor as well as a scaffold factor for the uniporter (78–81). Loss of MCUR1 leads to reduced mitochondrial Ca^{2+} uptake, and in some, though not all studies, to reduced assembly and faster turnover of Complex IV (79). MCUR1 has also been associated with Ca^{2+}-sensing for the permeability transition, and yeast homologs appear to be involved in proline metabolism (79, 82). The mechanism for these various functions remains somewhat mysterious.

6.5. Supercomplex

Complex I, III, and IV are known to exist in a supercomplex form (83, 84). Whether this supercomplex exerts functional effects on energetic flux through the ETC or reflects optimal packing in mitochondrial cristae remains controversial (85). A very recent study explores Ca^{2+} regulation of supercomplexes in a sex-dependent manner, finding that female rat and healthy human donor ventricular cardiomyocytes displayed lower uniporter, mitochondrial Ca^{2+}, and ROS levels compared to male counterparts though myocardial O2 consumption was unchanged(86). Intriguingly, females appeared to have increased amounts of complexes I, III, and IV assembled into supercomplexes. This depended on the amounts of the estrogen-dependent Complex IV subunit COX7RP, a peptide that sits

at the interface between Complex III and IV and promotes supercomplex assembly and respiratory efficiency (87, 88). Increasing COX7RP led to reduced mitochondrial Ca^{2+} and ROS, whereas inhibiting its expression produced the opposite effects. The combination of increased supercomplex assembly and reduced mitochondrial Ca^{2+} and ROS was felt to lower the propensity to arrhythmias in pre-menopausal females (Fig 2B).

6.6. ATP Synthase

The F_1-F_O-ATP Synthase utilizes the $\Delta\Psi$ produced by the ETC to coupling H^+ influx to ATP synthesis from ADP and Pi (89, 90). It is a large (500 kDa) multi-subunit protein mainly divided into the membrane-embedded F_O and soluble F_1 rotary domains. The two domains are connected by central and side stalks. The catalytic F1 domain protrudes into the matrix and consists of 3 α- and 3 β-subunits and a γδε-subunit, with catalytic nucleotide binding sites at the α- and β-subunit interfaces. Within the F_O domain, the c-subunit is a highly conserved 75 residue hydrophobic peptide that arranges its 10–12 oligomers into a ring formation, creating the machinery coupling proton movement to rotary movement (91). Although, Ca^{2+} may bind components of the ATP synthase and alter its behavior during pathophysiological states (see section 7.3 below), direct allosteric regulation has not been shown during normal physiology. Nevertheless, as described above, multiple studies have revealed that Ca^{2+}-induced changes in substrate preference, TCA cycle, and $\Delta\Psi$ culminate in a two-fold increase in ATP synthesis. Intriguingly, a recent study in trypanosomal parasites showed an interaction between MCU and the membrane-embedded c-subunit of the ATP synthase, while genetic inhibition of trypanosomal MCU reduced ATP production (92). Although an interaction was seen between human MCU and the c-subunit in heterologous systems in that study, confirmation of the interaction in intact mammalian mitochondria or potential functional consequences were not explored. Especially as MCU dimers align in areas of convex membrane curvature (16), contrasted with the convex membrane curvature induced within cristae by ATP synthase dimers, a subpopulation of uniporter channels interacting with the ATP synthase may alter the local environment and modify ATP production.

7. Ca^{2+} regulation of the ETC and F_1-F_O-ATP synthase in pathophysiology.

7.1. Complex I impairment triggers compensatory increases in mitochondrial Ca^{2+} to maintain energetic homeostasis.

Genetically-encoded defects in the OXPHOS machinery belong to a collective group of disorders called mitochondrial diseases. Although rare, mitochondrial disease is the most common in-born error in metabolism occurring in 1 in 5,000 live births, most commonly affecting Complex I of the ETC (93, 94). These diseases can present with a variety of pathologies, often affecting organs with high energetic demand, such as the nervous system, liver, skeletal muscle, and heart. Study of animal and cell models of these diseases have revealed striking new forms of Ca^{2+} regulation.

During ETC impairment in mitochondrial diseases, our group revealed that increased mitochondrial Ca^{2+} is a compensatory mechanism needed for survival (95). Previous studies investigating mitochondrial Ca^{2+} during ETC dysfunction typically found reduced

or unchanged Ca^{2+} uptake and impaired $\Delta\Psi$ (96–101). Because the level of Ca^{2+} influx is heavily dependent on $\Delta\Psi$, a change to either the $\Delta\Psi$ or uniporter activity will alter the size of Ca^{2+} influx and one may mask changes in the other. To overcome confounding results by changes in $\Delta\Psi$, pH, or other factors, we used voltage-clamp electrophysiology on mitoplasts (mitochondria devoid of its outer membrane) to fully control $\Delta\Psi$, matrix and external solutions. Under these controlled conditions, we found that impairing ETC results in substantial increases in uniporter Ca^{2+} currents. In fact, closer examination of mitochondria from multiple models of ETC or Complex I impairment reveal an early increase in mitochondrial Ca^{2+} uptake and uniporter levels, seen by others as well (68, 102). This increase in mitochondrial Ca^{2+} served to rescue respiration and ATP synthesis to near wild-type levels in animal models of mitochondrial cardiomyopathies (95). The mechanism for this was abrogation of CLIPT, described above in the section on Complex I (section 6.1). Under normal conditions, MCU levels were kept low by transient interactions with Complex I leading to uniporter degradation. However, when Complex I was impaired in these mitochondrial diseases, there was loss of the MCU-Complex I interaction and a striking and sustained increase in uniporter channel levels. Increased channel stability is a post-transcriptional homeostatic mechanism to preserve energetic flux through the remaining ETC. This mechanism was evident across all cell types tested and across species including *Drosophila*, mice, and humans. In fact, in *Drosophila* with Complex I deficiency, survival was dependent on the uniporter. Inhibiting uniporter expression caused catastrophic mortality during development, whereas boosting uniporter expression or stability rescued survival to near wild-type levels. This reveals not only the importance of mitochondrial Ca^{2+} during Complex I dysfunction, but also suggests potential therapeutic targets for these diseases.

7.2. Increases in mitochondrial Ca^{2+} during hypoxia indirectly limit CoQ diffusion between Complex II and Complex III.

Flux through the Q-cycle of Complex III is thought to depend on the speed of CoQ diffusion from upstream ETC complexes. A recent study elegantly demonstrated how Ca^{2+} can indirectly regulate CoQ diffusion during pathological states (103). Specifically, during hypoxic injury, Complex I enters a deactivated state which decreases the matrix pH. Within the matrix, Ca^{2+} is often stored in dense calcium phosphate granules, and matrix acidification is conducive to freeing Ca^{2+} from these granules. In turn, the increased matrix Ca^{2+} triggers Ca^{2+} extrusion through NCLX, which brings more Na^{+} in. The sudden increase in matrix Na^{+} during hypoxia was found to decrease the fluidity of the inner mitochondrial membrane, slowing CoQ diffusion from Complex II to Complex III. Ultimately, this causes OXPHOS to slow at this point of the ETC (Fig. 2C). With limited $CoQH_2$ diffusion, the semiquinone already bound within Complex III cannot effectively participate in the Q cycle and excess electrons leak out as potentially toxic superoxide. Thus, unlike the beneficial effects of elevated matrix Ca^{2+} during Complex I impairment (section 7.1), acute elevations in Ca^{2+} during hypoxic injury may promote the production of harmful ROS through Complex III.

7.3. Ca^{2+} binding to the F1 subunit of the ATP synthase may help trigger the permeability transition.

Despite the various physiological roles of Ca^{2+} listed previously, it has been appreciated for >75 years that excess Ca^{2+} is deleterious for mitochondrial function (104). Mitochondrial Ca^{2+} overload opens a mysterious, cyclosporine-sensitive, large-pore channel known as the permeability transition pore (PTP), which leads to catastrophic mitochondrial depolarization, swelling, and structural disruption (105–108). In many cases, this can trigger cell death.

A current leading theory regarding the nature of the PTP is that it occurs via a pathological conformational change in the ATP synthase (109–112). This is based on the finding that a Ca^{2+}-dependent high-conductance channel forms. The data described above suggesting that MCU and the ATP Synthase *c*-subunit interact in trypanosomes, and perhaps mammals, may further support this theory (92). Mechanistically, the current hypothesis focuses the catalytic site between α and β subunits of the soluble F_1 component of the ATP synthase (113). This site typically uses Mg^{2+} to coordinate ATP or ADP during the synthesis cycle around the central rotor shaft. The current hypothesis posits that at high concentrations, Ca^{2+} replaces Mg^{2+} at this nucleotide binding pocket, triggering downstream changes that open a channel within the transmembrane F_O component of the ATP synthase (Fig. 2D) (108). In an alternative mechanism, other investigators posited that Ca^{2+} can trigger the c-subunits to spontaneously fold into a β-sheet conformation that has ion transport activity and resembles amyloidogenic proteins (114). However, because this transition requires extremely high, non-physiological Ca^{2+} concentrations, (1 mM) its occurrence in intact mitochondria remains questionable.

7.4. Ca^{2+} regulation of the adenine nucleotide translocator has not been well established.

A problem with the ATP synthase hypothesis for the PTP has been evidence showing the presence of residual PTP-like channels when multiple ATP synthase components have been deleted (115–117). This has led to the resurfacing of an older hypothesis suggesting the existence of a separate class of PTP channels encoded by the adenine nucleotide translocator (ANT) (118), as deletion of multiple ANT isoforms also drastically reduces PTP activity (119) and possible channel-like pores have been seen with crosslinking proteomics and electrophysiology (120, 121). Unfortunately, currently there is little evidence revealing a potential mechanism for Ca^{2+}-induced ANT rearrangement into a PTP-like channel.

8. Future Directions

First, the current literature provides insights on a range of different tissues such as liver, heart, and skeletal muscle, but there is a clear gap in understanding whether these mechanisms are entirely conserved across organs, or whether other novel tissue-specific mechanisms of Ca^{2+}-dependent OXPHOS regulation exist. Second, although data from tissue- or whole-animal knockout studies has provided substantial insight into Ca^{2+} regulation of metabolism, the ability to acutely perturb mitochondrial Ca^{2+} levels to investigate how OXPHOS is affected during physiological activities has been hampered by the lack of highly specific compounds that can be given to intact cells or organisms. Drug screens may hopefully identify novel tools to further investigate the impact of

mitochondrial Ca^{2+} fluxes (122–124). Finally, many studies of metabolism rely on steady-state or endpoint measurements. These assays only partly capture significant changes in metabolic flux through pathways such as metabolite transport, TCA cycle, and the ETC, that are impacted by Ca^{2+} regulation. The advent of new technologies using isotope-labelled tracers for quantitative flux analysis may offer new ways to address this limitation, allowing more granular assessment of how Ca^{2+} may alter fluxes and enzymatic activity (125). In addition, as sensors for Ca^{2+}, ATP, NAD^+, and other metabolites improve, we will have the opportunity to carry out multi-modal assessments simultaneously, to see how these parameters interact.

9. Conclusion.

Compartmentalizing Ca^{2+} within the cell is an essential attribute for Ca^{2+} signaling. It is clear that mitochondrial Ca^{2+} is necessary for normal function and especially during stress or work while Ca^{2+} overload can result in disease and cell death. Ca^{2+} is imported through the uniporter, utilizing the $\Delta\Psi$ created by the ETC, and exported through NCLX, LETM1, and TMBIM5. In normal physiology, it is clear that mitochondrial Ca^{2+} (1) augments ATP production by stimulating NADH synthesis through the TCA cycle and (2) shifts preference towards glycolysis by its stimulatory effects on PDH flux. Although Ca^{2+} has few direct allosteric effects on the ETC and ATP synthase, a newer paradigm is arising suggesting that Ca^{2+} and its transporters are crucial indirect regulators of OXPHOS by altering the stability, assembly, and conformation of these complexes.

Our understanding of mitochondrial Ca^{2+} in the context of OXPHOS defects is still expanding. These recent discoveries of new binding partners between ETC complexes and Ca^{2+} regulators are providing more insight into how cells manage mitochondrial Ca^{2+}, especially during stress. Because mitochondrial damage is a central feature of pathophysiology across many organs and diseases, expanding our understanding of how Ca^{2+} regulates OXPHOS and may worsen or compensate for ETC dysfunction will have therapeutic implications for the future.

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ABBREVIATION

MCU	Mitochondrial calcium uniporter
ETC	Electron transport chain
IMM	Inner mitochondrial membrane
OXPHOS	Oxidative phosphorylation
TCA	Tricarboxylic acid cycle

ROS	Reactive Oxygen Species
PDH	Pyruvate Dehydrogenase
IDH	Isocitrate Dehydrogenase
a-KGH	α-ketoglutarate dehydrogenase
PTP	Permeability transition pore
ER	Endoplasmic reticulum
MCU	Mitochondrial calcium uniporter, pore-forming subunit
MICU1-3	Mitochondrial uptake 1–3
MCUb	Mitochondrial calcium uniporter dominant negative subunit beta
EMRE	Essential component of the mitochondrial calcium uniporter

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PERSPECTIVES

- Mitochondrial Ca^{2+} has long been known to be an allosteric activator of metabolite transporters, pyruvate-to-acetyl CoA conversion, and tricarboxylic acid (TCA) cycle dehydrogenases, which ultimately increase ATP synthesis two-fold.
- Recent data has shown that mitochondrial Ca^{2+} is a critical regulator of fuel substrate utilization. Reducing mitochondrial Ca^{2+} influx produces a profound shift away from glycolysis towards fatty acid oxidation.
- Recent investigations have expanded the mechanism of Ca^{2+} regulation beyond direct allosteric effects to indirect mechanisms that alter the stability and function of OXPHOS complexes, particularly Complex I. These novel forms of regulation may be exploited therapeutically.

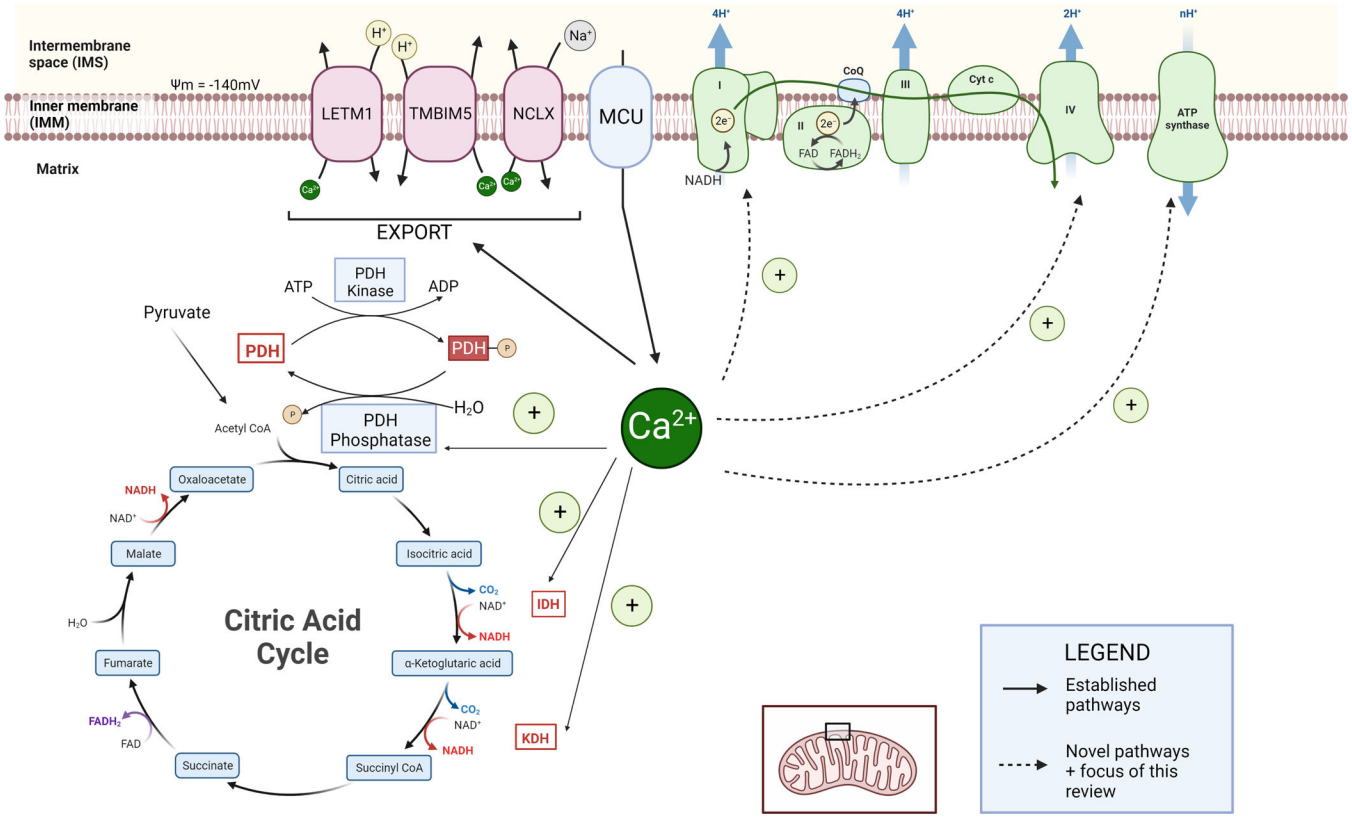

Figure 1 –. ETC and Ca^{2+} signaling overview.

MCU imports Ca^{2+} which is known to activate the following enzymes: PDH phosphatase, Isocitrate dehydrogenase (IDH), and α-ketoglutarate dehydrogenase (AKGDH). Export mechanisms include LETM1, TMBIM5, and NCLX. Known pathways are shown in solid arrows. Newer mechanisms involve Complex I, CoQ, complex IV, and ATP Synthase, shown in dashed arrows.

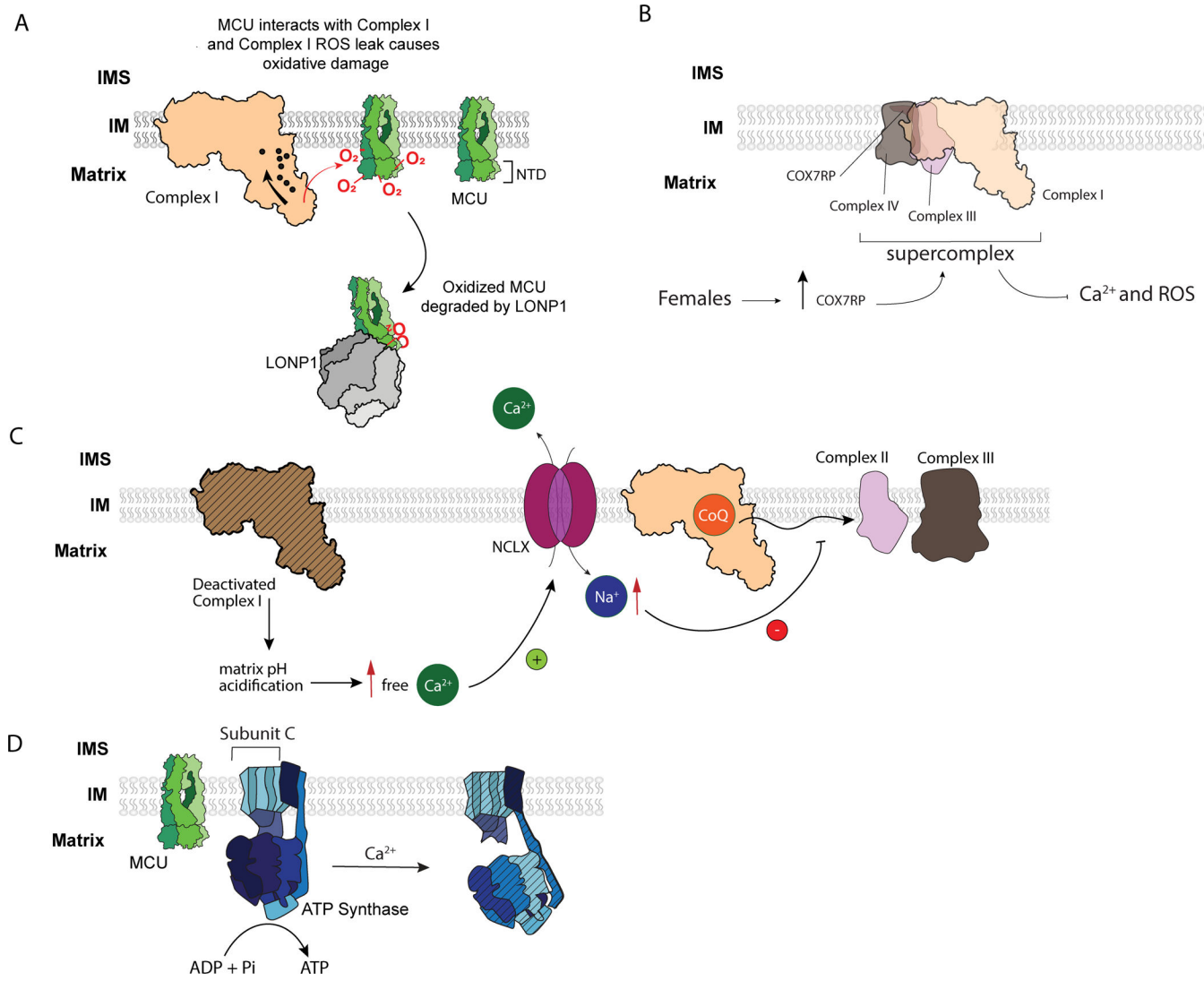

FIGURE 2 –. New mechanisms involving mitochondrial Ca^{2+}.

(A) CLIPT – Complex I interacts with MCU and maintains its protein turnover rate. (Adapted from ref. (68))

(B) Complex IV subunit COX7RP is estrogen dependent, sits at the interface between Complex III and IV, and promotes supercomplex assembly and respiratory efficiency. Increasing COX7RP led to reduced mitochondrial Ca^{2+} and ROS, whereas inhibiting its expression produced the opposite effects (see ref. (86)).

(C) Deactivated complex I promotes matrix acidification which dissolve CaP precipitates. The increased matrix Ca^{2+} triggers NCLX to exchange Ca^{2+} for Na^{+}. Increased matrix Na^{+} decreases IMM fluidity and prevents CoQ diffusion, decreasing oxphos at Complex II + III. (Adapted from ref. (103))

(D) MCU binds to the subunit c of ATP Synthase in *Trypanosoma* (see ref. (92)). Excess Ca^{2+} may trigger rearrangement of the F_1 component of the ATP synthase to produce channels in the membrane component (see ref. (108)).