

Crosstalk signaling between mitochondrial Ca^{2+} and ROS

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1. ABSTRACT

Mitochondria are central to energy metabolism as the source of much of the cell's ATP, as well as being a hub for cellular Ca^{2+} signaling. Mitochondrial Ca^{2+} is a positive effector of ATP synthesis, yet Ca^{2+} overload can lead to mitochondrial dysfunction and cell death. Moreover, Ca^{2+} uptake by mitochondria is involved in shaping cellular Ca^{2+} dynamics by regulating the concentrations of Ca^{2+} within microdomains between mitochondria and sarco/endoplasmic reticulum and plasma membrane Ca^{2+} transporters. Reactive oxygen species (ROS) generated as a consequence of ATP production in the mitochondria are important for cellular signaling, yet contribute to oxidative stress and cellular damage. ROS regulate the activity of redox sensitive enzymes and ion channels within the cell, including Ca^{2+} channels. For both Ca^{2+} and ROS, a delicate balance exists between the beneficial and detrimental effects on mitochondria. In this review we bring together current data on mitochondrial Ca^{2+} uptake, ROS generation, and redox modulation of Ca^{2+} transport proteins. We present a model for crosstalk between Ca^{2+} and ROS signaling pathways within mitochondrial microdomains.

2. INTRODUCTION

In the past decade, mitochondria have become a focal point of modern biomedical research due in part to the central role the organelle plays in numerous pathologies including, but not limited to cardiovascular disorders such as atherosclerosis, ischemic heart disease, ischemia-reperfusion injury, and cardiac failure, neurodegenerative disorders related to mitochondrially derived oxidative stress such as Huntington's disease, Parkinson's disease, Alzheimer's disease, as well as playing a role in the aging process and diabetes (1-7).

The main function of mitochondria is ATP production, which occurs during mitochondrial oxidative phosphorylation (ox-phos). During ox-phos, electrons from reduced substrates are transferred to O_2 through a chain of respiratory electron transporters including the complex I, III, and IV H^+ pumps, which in turn generate a proton gradient across the mitochondrial inner membrane. The electrochemical energy of this gradient is then used by the ATP synthase (complex V) which couples H^+ reuptake with ADP phosphorylation in the matrix to generate ATP.

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Electrons however, may leak from reduced sites in the respiratory chain and react with oxygen to form reactive oxygen species (ROS) which play an important role in cell signaling, but are better known for creating oxidative stress (8).

In several cell types, mitochondria also serve as a very efficient Ca^{2+} buffer, taking up substantial amounts of cytosolic Ca^{2+} at the expense of mitochondrial membrane potential ($\Delta\Psi_m$). The pathways of Ca^{2+} entry into mitochondrial matrix are known as the mitochondrial calcium uniporter (MCU) (9), the “rapid mode” mechanism (10), and the mitochondrial ryanodine receptor (11). The main role of mitochondrial Ca^{2+} is the stimulation of the ox-phos enzymes (12). In addition to ox-phos, mitochondria are central players in cellular Ca^{2+} signaling by shaping and buffering cellular Ca^{2+} signals (13-15).

As a consequence of Ca^{2+} uptake, mitochondria can suffer Ca^{2+} overload, triggering the opening of the permeability transition pore (PTP) which is associated with apoptosis *via* the mitochondrial pathway or necrosis due to mitochondrial damage (16). The PTP has been shown to be promoted by thiol oxidation and inhibited by antioxidants, lending support for a role of ROS in pore opening (17). Furthermore, it has been demonstrated that mitochondrial Ca^{2+} uptake can lead to free radical production (18, 19). From a thermodynamic point of view, however, it has been noted that Ca^{2+} uptake occurring at the expense of membrane potential should result in a decrease in ROS production (20). The mechanism for Ca^{2+} -induced ROS generation is not understood and is a topic of great debate in the field.

A delicate balance exists between moderate ROS production to modulate physiological signaling and overproduction of ROS that ultimately leads to oxidative stress. ROS detoxification pathways exist to minimize oxidative damage, but insults that lead to excessive ROS production precipitate changes in cellular redox homeostasis, including changes in Ca^{2+} handling and further alterations of ROS production. It is clear that a complex interdependence exists between mitochondrial energy production, Ca^{2+} uptake, ROS generation, ROS detoxification, and redox signaling. In this review, we will discuss each of these points independently, and then examine how crosstalk between mitochondrial Ca^{2+} handling and ROS generation play a role in normal cell function, and how deviations from normal signaling lead to mitochondrial dysfunction, and ultimately, cell death.

3. MITOCHONDRIAL Ca^{2+} DYNAMICS

The driving force for Ca^{2+} uptake by mitochondria is provided by an electrochemical membrane potential ($\Delta\Psi_m$) across the inner mitochondrial membrane (IMM). The $\Delta\Psi_m$ is established by outward proton pumping from the matrix via the respiratory electron transport chain (ETC) H^+ pumps. The outer mitochondrial membrane (OMM) is made permeable to Ca^{2+} via the voltage dependant anion channel (VDAC) which regulates the movement of Ca^{2+} to the intermembrane space (IMS)

(21). The relatively impermeable IMM contains multiple mechanisms responsible for both Ca^{2+} influx and efflux (for extensive review of mitochondrial Ca^{2+} transporters, see (22, 23)).

3.1. Mitochondrial Ca^{2+} mobilization: uptake and efflux

Uptake via the mitochondrial calcium uniporter (MCU) is considered the primary route of Ca^{2+} influx in the mitochondria (see Figure 1 for a schematic of mitochondrial Ca^{2+} uptake and efflux mechanisms). Recent patch clamp studies have determined that the MCU is a highly selective Ca^{2+} channel with a $K_d < 2\text{nM}$ (24). The MCU is blocked by ruthenium red (RuR) and a colorless ruthenium red derivative, Ru360 (25, 26). The MCU has activation and a transport site for Ca^{2+} , thus exhibiting second order kinetics (27-29). Despite the wealth of pharmacologic and electrophysiological data describing the MCU, the molecular identity of the channel is still unknown. Attempts to identify the MCU have led to the isolation of a number of soluble Ca^{2+} binding glycoproteins from ox and rat liver and beef heart mitochondria (30-32). Depletion of a 34-40 kDa glycoprotein from the mitochondria IM space reduced Ca^{2+} transport that was partially restored upon re-addition of the glycoprotein fraction. Subsequently, antibodies raised against purified glycoproteins inhibited MCU Ca^{2+} transport (33, 34). A recent study has suggested that mitochondrial uncoupling proteins (UCP2 and UCP3) may comprise or play a role in MCU Ca^{2+} transport as well (35).

A second mechanism of Ca^{2+} uptake in mitochondria is the rapid mode (RaM) described by Sparanga *et al.* (10). RaM facilitates very rapid Ca^{2+} influx at the onset of a $[\text{Ca}^{2+}]_c$ pulse, and is subsequently inhibited by binding of Ca^{2+} from the pulse at an external binding site. This mechanism may provide mitochondria with a burst of Ca^{2+} to facilitate an immediate activation of Ca^{2+} dependent processes without a possible delay from slower MCU mediated influx. In accord with the frequency of Ca^{2+} pulses in different tissues, the RaM in liver and heart mitochondria differ in their regulation. In heart cells in which excitation-contraction generated Ca^{2+} pulses are rapid, at least one minute is required to reset the RaM prior to subsequent reactivation, whereas the liver RaM resets in a fraction of a second after the local $[\text{Ca}^{2+}]_c$ drops to 100 nM (36). As with the MCU, the molecular identity of RaM is unknown.

The identification of a ryanodine receptor (RyR) in the IMM of cardiac mitochondria represents a third mechanism of mitochondrial Ca^{2+} import (mRyR) (11, 37, 38). The mRyR identified in rat heart mitochondria was characterized using subtype specific antisera as RyR1 (skeletal muscle subtype) (38). Via ryanodine titration, it was determined that mRyR is activated at low concentrations of Ca^{2+} with a peak open channel probability at 10-30 μM Ca^{2+} , similar to skeletal muscle RyR1 (11). Purified mRyR channels were found to have characteristics similar to those of the skeletal muscle SR-RyR1 at the single channel level (37). High affinity Ca^{2+} transport by the mRyR suggest a mechanism to sequester

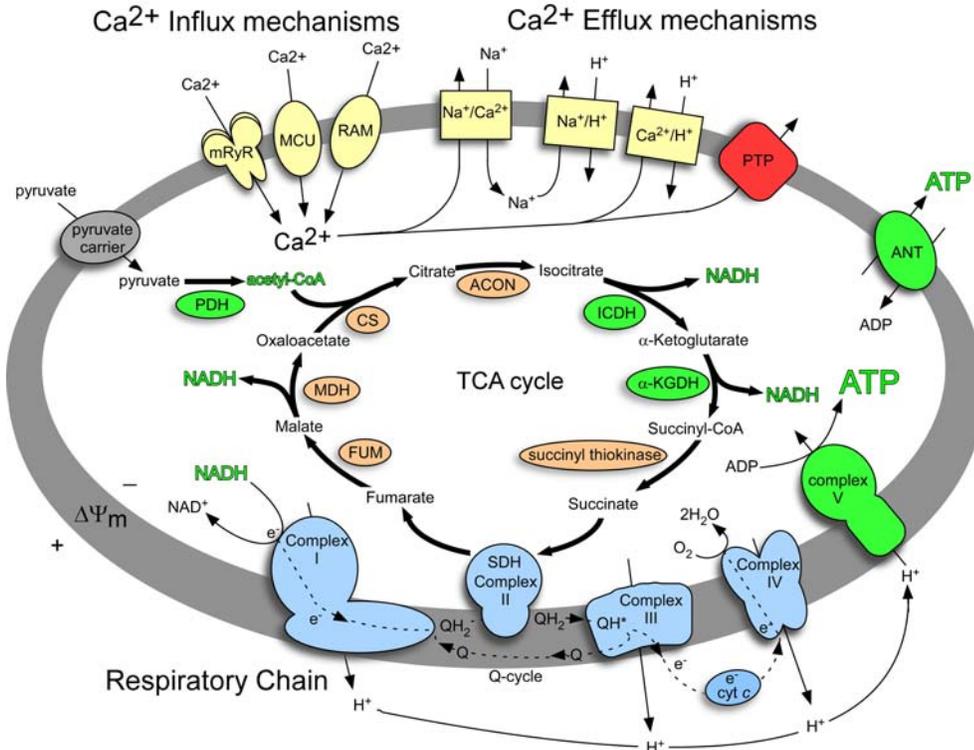


Figure 1. Mitochondrial Ca^{2+} dynamics and stimulation of the TCA cycle and oxidative phosphorylation. Metabolite and ion transport are represented by thin arrows passing through membrane carriers, metabolic pathways by thick arrows, respiratory chain electron transport by broken arrows, and TCA cycle enzymes stimulated by Ca^{2+} are displayed in yellow. Mitochondrial Ca^{2+} influx and efflux mechanisms are displayed in green, and the respiratory chain is displayed in blue. The mitochondrial outer membrane has been omitted for clarity. MCU, mitochondrial calcium uniporter; mRyR, mitochondrial ryanodine receptor; RAM, rapid mode; PTP, permeability transition pore; ANT, adenine nucleotide transporter; PDH, pyruvate dehydrogenase; CS, citrate synthase; ACON, aconitase; ICDH, isocitrate dehydrogenase; α -KGDH, α -ketoglutarate dehydrogenase; SDH, succinate dehydrogenase; FUM, fumarase; MDH, malate dehydrogenase; $\Delta\Psi_m$, membrane potential.

Ca^{2+} on a beat-to-beat basis in cardiac cells. Accordingly, mitochondrial Ca^{2+} uptake occurs on a beat for beat basis in cardiomyocytes (39-42). Thus, cardiac mitochondria can match excitation-contraction derived Ca^{2+} signals to increased ATP production in order to meet the high energy demand of the heart, a mechanism termed excitation-metabolism coupling (11).

Mitochondria have a finite capacity for Ca^{2+} . In the presence of inorganic phosphate, mitochondrial calcium phosphate precipitates form, facilitating total accumulation of Ca^{2+} approaching 1M while maintaining low $[\text{Ca}^{2+}]_m$ (43, 44). This extraordinary capacity allows mitochondria to form a significant cellular Ca^{2+} buffer, more so than the ER and SR. To this end, mitochondrial Ca^{2+} overload can occur, leading to failure and eventual cell death without an effective Ca^{2+} export mechanism. Two mechanisms exist for the controlled export of Ca^{2+} from mitochondria, a Na^+ dependent and a Na^+ independent exchanger (Figure 1). Mitochondrial Ca^{2+} efflux has been comprehensively reviewed (45, 46). Briefly, the predominant Ca^{2+} efflux mechanism in excitable cells such as heart, brain, and skeletal muscle is the Na^+ dependant $\text{Na}^+/\text{Ca}^{2+}$

($\text{Na}^+/\text{Ca}^{2+}$). $\text{Na}^+/\text{Ca}^{2+}$ is an electrogenic antiporter with a stoichiometry of transport of 3:1 ($\text{Na}^+:\text{Ca}^{2+}$) (47, 48). The Na^+ imported into the mitochondria in exchange for Ca^{2+} is subsequently exchanged for H^+ via the Na^+/H^+ antiporter, thus Ca^{2+} efflux comes at the cost of $\Delta\Psi_m$. The alternative mechanism, a $2\text{H}^+/\text{Ca}^{2+}$ exchanger ($2\text{H}^+/\text{Ca}^{2+}$) is predominantly expressed in liver, kidney, lung, smooth muscle and is an electroneutral transporter (49-51). Both $\text{Na}^+/\text{Ca}^{2+}$ and $2\text{H}^+/\text{Ca}^{2+}$ have been found in mitochondria from all tissues examined indicating Ca^{2+} efflux is not mediated by a single mechanism in any given cell type. In energized mitochondria, a steady-state Ca^{2+} “set point” exists that is established by matched Ca^{2+} influx and efflux (52). The permeability transition pore (PTP, discussed in detail below 3.3. Ca^{2+} overload: The permeability transition and apoptosis) has also been suggested to act as a Ca^{2+} release mechanism by transiently opening and releasing Ca^{2+} to the cytoplasm (53, 54).

3.2. Ca^{2+} regulation of mitochondrial metabolism

ATP production via oxidative phosphorylation is the primary function of mitochondria, and Ca^{2+} is the hallmark stimulatory signal for activation of numerous

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mitochondrial enzymes (55-57). Physiological increases in $[\text{Ca}^{2+}]_m$ lead to the allosteric activation of TCA cycle enzymes including isocitrate dehydrogenase and α -ketoglutarate dehydrogenase, as well as pyruvate dehydrogenase (57). The net effect of TCA cycle activation is a boost in reduced ox-phos substrate synthesis (NADH and FADH), enhanced respiratory chain activity and a subsequent increase in H^+ pumping. In experiments that monitored the redox state of NADH/NAD⁺ and FADH₂/FAD, it was shown that following Ca^{2+} uptake, $\Delta\Psi_m$ dropped and pyridine nucleotide/ flavoprotein pools became transiently oxidized as ox-phos was stimulated to restore $\Delta\Psi_m$ (58, 59). Ca^{2+} pulses also stimulate the adenine nucleotide transporter (60) and Complex V (mitochondrial F_0F_1 ATP synthase) (61), harnessing the H^+ gradient to up-regulate ATP production. As well as stimulating ox-phos, Ca^{2+} also stimulates α -glycerolphosphate dehydrogenase (62), a component of the glycerol phosphate shuttle that supplies NAD⁺ for glycolysis.

3.3. Ca^{2+} overload: the permeability transition pore and apoptosis

There are two sides to the effects of Ca^{2+} on mitochondrial function. On the beneficial side, Ca^{2+} is a positive effector of ox-phos. However, when overloaded, Ca^{2+} becomes a pathological signal leading to opening of the PTP and the subsequent initiation of apoptosis (63). The PTP is composed of a number of mitochondrial proteins in both the IMM and OMM that associate to form a large conductance channel that, when opened, dissipates $\Delta\Psi_m$ and allows matrix solutes < 1.5 kDa and Ca^{2+} to be released from the mitochondria (64-66). The primary factors that are thought to compose the PTP are the OMM VDAC, the IMM ANT, and the soluble matrix protein cyclophilin D (67). However, there appears to be a great deal of variability, as the PTP has been shown to function in the absence ANT1 and ANT2 in double-knockout mice (68) as well as in the absence of cyclophilin D (69). ANT and VDAC form contact sites between the IMM and OMM that often associates with and are modulated by additional proteins (70) including hexokinase (65), the mitochondrial benzodiazepine receptor (71), Bax (72), and creatine kinase (67). PTP opening can be inhibited by cyclosporine A, which binds to matrix cyclophilin D, preventing its association with ANT. Ca^{2+} overload is the primary stimulator of the opening of the PTP, but other stimuli are involved in sensitizing the PTP such as oxidative stress (eg. ROS, oxidized GSH and pyridine nucleotide pools) (45, 73, 74), ADP/ATP depletion (with a concurrent rise in matrix Pi) (75, 76). Likewise, ADP, a reduced GSH and pyridine nucleotide pool, and acidic pH (77) are all inhibitors of PTP opening. A model proposed by He and Lemasters suggests that clusters of proteins that have been modified by oxidative damage can form unregulated pores in the mitochondrial inner membrane (78). These protein cluster pores are in turn blocked by mitochondrial chaperones and cyclophilin D. This model provides a possible explanation for the variability of PTP components by proposing that the blocked pores are regulated by the sensitizers and inhibitors described above.

Opening of the PTP leads to mitochondrial depolarization, loss of matrix solutes including GSH, pyridine nucleotides, ADP/ATP, and leads to the release of cytochrome *c* from the intermembrane space (comprehensively reviewed in (53, 63)). Cytochrome *c* release is required for caspase activation that initiates the apoptotic program (79). In isolated mitochondria, Ca^{2+} induced opening of the PTP results in mitochondrial swelling and release of IMS contents as the OMM ruptures and mitoplasts expand (80). Although the PTP is a leading pathway leading to cytochrome *c* release (81), PTP is not necessary for apoptosis as there are reports of non-PTP cytochrome *c* release (82, 83). The actual mechanism (s) for *in vivo* cytochrome *c* release are not known.

PTP opening is not always an all-or-nothing event. Transient opening, or flicker, of the PTP has been observed in many cell types (84, 85), as well as in isolated mitochondria (54). The frequency of transient PTP opening is primarily determined by free matrix $[\text{Ca}^{2+}]$ (76, 86). Physiological PTP flicker has been suggested to be a mechanism for the release of Ca^{2+} from overloaded mitochondria (53, 54, 87, 88). In this manner, PTP flicker serves as a physiological safety valve to prevent Ca^{2+} overload, mitochondrial failure, and cell death.

4. Ca^{2+} AND MITOCHONDRIAL REACTIVE OXYGEN SPECIES

Mitochondrial oxidative stress is caused by an imbalance between ROS generation and ROS detoxification. There are physiological benefits to controlled ROS generation such as cell signaling (8), but unregulated, ROS can lead to oxidative stress, cellular damage, and eventually cell death. In the following section, mitochondrial sources of ROS and the defense mechanisms that maintain a physiological oxidative homeostasis will be discussed, as well as the role Ca^{2+} plays in ROS generation.

4.1. Reactive oxygen species

Reactive oxygen species are typically defined as molecules or ions formed by the incomplete one-electron reduction of oxygen. Categorically, ROS include free radicals such as superoxide, hydroxyl radical, and singlet oxygen, as well as non-radical species such as hydrogen peroxide. Oxygen free radicals are highly reactive and have the capacity to damage cellular components such as proteins, lipids, and nucleic acids. Mitochondria are considered a major source of ROS in the cell. In liver, the total rate of H_2O_2 production has been calculated to be on the order of 90 nmol/min/g tissue wet weight, mostly of which could be attributed to mitochondrial generation (89, 90). The respiratory chain complexes I and III are the primary mitochondrial sources of univalent reduction of O_2 into superoxide (O_2^-) (91-94). O_2^- is very unstable and quickly reacts with nearby molecules, naturally dismutates into hydrogen peroxide (H_2O_2) and O_2 , or is enzymatically dismutated by the superoxide dismutase (SOD) enzyme family. In contrast to O_2^- , H_2O_2 is membrane permeable. H_2O_2 can in turn react with metal ions to form the highly reactive hydroxyl radical (OH) via the Fenton reaction.

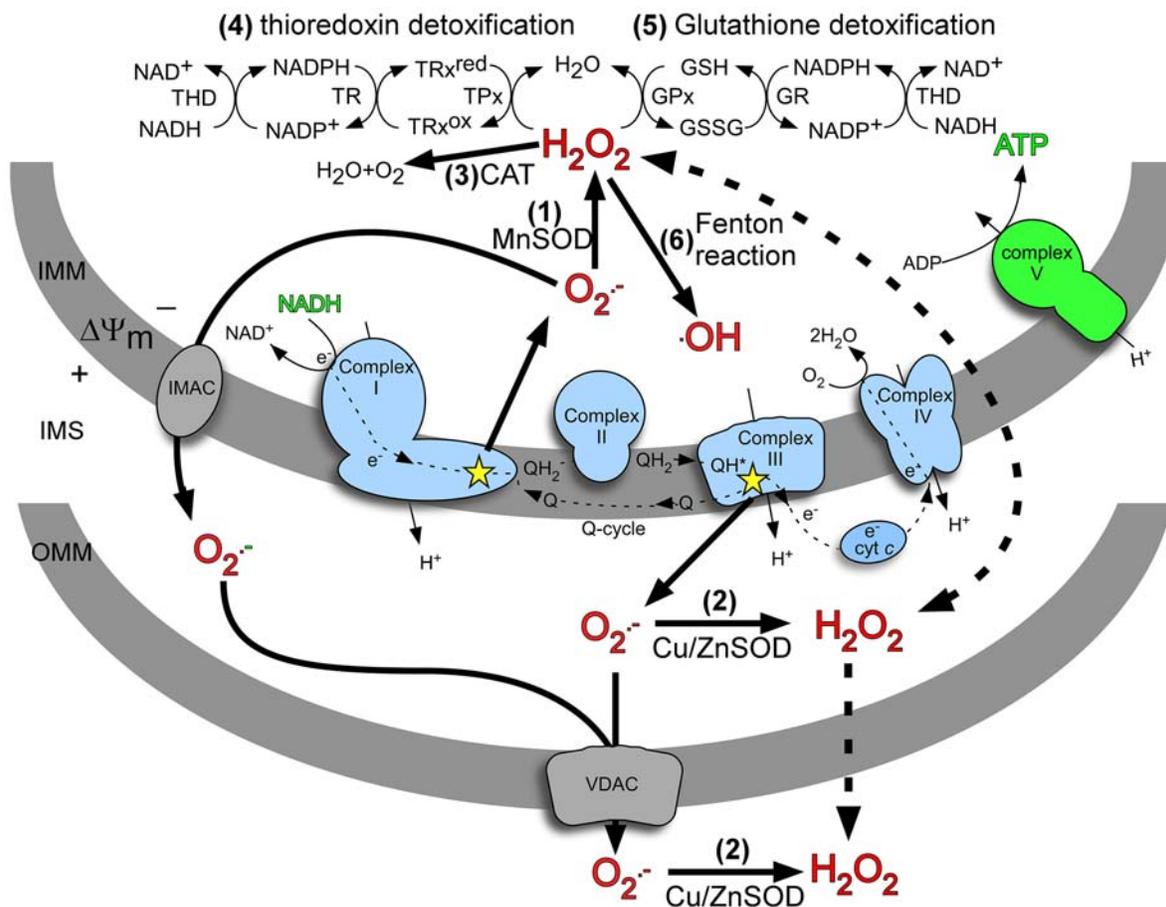


Figure 2. Mitochondrial ROS production and defense. Superoxide ($\text{O}_2^{\cdot-}$) generated by the respiratory chain is mostly released to the matrix at complex I and the IMS at complex III (indicated by stars). $\text{O}_2^{\cdot-}$ can naturally dismutate to hydrogen peroxide (H_2O_2) or is enzymatically dismutated by matrix MnSOD (1) or Cu/ZnSOD (2) in the IMS or cytosol. H_2O_2 is detoxified in the matrix by catalase (3), the thioredoxin/thioredoxin peroxidase system (4), or the glutathione/glutathione peroxidase system (5). Alternately, H_2O_2 can react with metal ions to generate the highly reactive hydroxyl radical ($\cdot\text{OH}$) via Fenton chemistry (6). $\text{O}_2^{\cdot-}$ is not membrane permeable but can pass through ion channels (solid lines), whereas H_2O_2 can pass freely through membranes (dashed lines). IMM, inner mitochondrial membrane; IMS, intermembrane space; OMM, outer mitochondrial membrane; $\text{O}_2^{\cdot-}$, superoxide; H_2O_2 , hydrogen peroxide; MnSOD, manganese superoxide dismutase; Cu/ZnSOD, copper/zinc superoxide dismutase; CAT, catalase; THD, NADH transhydrogenase; TR, thioredoxin reductase; TPx, thioredoxin peroxidase; TRx^{red} , reduced thioredoxin; TRx^{ox} , oxidized thioredoxin; GSH, glutathione; GSSG, glutathione disulfide; IMAC, inner membrane ion channel; VDAC, voltage dependant anion channel; $\Delta\Psi_m$, membrane potential.

4.2. ROS defense mechanisms

Cellular oxidative stress is the result in an imbalance between ROS generation and ROS detoxification. Cells employ a number of mechanisms to scavenge and detoxify ROS to maintain a permissive redox environment. Figure 2 details the mechanisms by which mitochondrially generated ROS are neutralized. The glutathione redox couple (GSH/GSSG) is the primary cellular redox buffer and is maintained at a very high ratio of reduced to oxidized glutathione (>30:1, GSH:GSSG) (95, 96). GSH is a cysteine containing tripeptide that can directly scavenge ROS or act as a cofactor for glutathione peroxidase which oxidizes glutathione to reduce H_2O_2 . GSH is subsequently reduced by glutathione reductase which uses NADPH as a substrate. In the mitochondria,

NADPH is generated at the expense of NADH by NADH transhydrogenase. The NADH/NAD⁺ redox couple also plays a role in redox buffering, but is maintained primarily in the oxidized (NAD⁺) form (97). Thioredoxin is a disulfide containing protein that can directly scavenge H_2O_2 as part of the thioredoxin reductase and thioredoxin peroxidase system (98, 99).

ROS detoxification enzymes including superoxide dismutase and catalase also play a direct role in ROS defense. Superoxide dismutase (SOD) catalyzes the dismutation of $\text{O}_2^{\cdot-}$ into H_2O_2 and O_2 . In mitochondria, SOD uses a manganese cofactor (MnSOD) while the predominantly cytosolic SOD uses copper and zinc (Cu/ZnSOD). ROS released into the mitochondrial matrix

is therefore neutralized by MnSOD (100). The fate of O_2^- in the IMS is short lived, as the superoxide anion within IMS may be quickly inactivated by Cu/ZnSOD which has been described in the IMS (101), or by cytochrome *c* which can be directly reduced by O_2^- and subsequently pass electrons to complex IV (102). H_2O_2 is detoxified by the enzyme catalase. It has been reported that Ca^{2+} can regulate cellular antioxidant defense systems by stimulating catalase and GSH reductase, interacting with calmodulin (CaM) which then interacts with enzymes involved in ROS homeostasis, or via release GSH early in Ca^{2+} induced PTP opening (12, 103). Ultimately, changes in redox homeostasis, especially at the level of the GSH/GSSG pool, can lead to oxidative damage, especially at the level of protein thiol oxidation by virtue of the high protein content in the IMM, leading to respiratory chain inhibition and further ROS generation (104).

4.3. ROS production at complex I

Complex I of the respiratory chain plays a major role in ROS production. Three mechanisms have been proposed to explain the mechanisms of ROS formation at this level, two of which are associated with forward electron flow through the respiratory chain complexes. Initial electron transport from NAD-linked substrates to complex I produce little O_2^- . Reduced sites within complex I including FMN (92), iron sulfur clusters (105) and semiquinones (106) have been suggested to be potential redox sites responsible for O_2^- production. Under conditions in which inhibitors that poison complex I function are absent, and in the presence of NAD⁺ linked substrates (pyruvate, glutamate and malate), O_2^- production is stimulated by high mitochondrial membrane potential ($\Delta\Psi_m$) generated by respiratory chain electrons transport (107). When mitochondria are energized via succinate oxidation (a complex II linked substrate), the rate of ROS production by complex I increase significantly. Under such conditions, O_2^- production by complex I is caused by a reverse net electron flow from succinate to NAD⁺. Reverse flow is enhanced by high $\Delta\Psi_m$ which underlies the rate of ROS production (108, 109). Mitochondrial Ca^{2+} uptake causes mild uncoupling of $\Delta\Psi_m$, hence, it should decrease ROS production as has been shown in other studies, investigating the role of mild uncoupling on mitochondrial ROS generation (20). Mitochondria from *Drosophila melanogaster*, exhibit 70% decrease of ROS generation from complex I upon drop of mitochondrial membrane potential of about 10 mV (110). This result is in agreement with the observation that proton leak mediated by uncoupling proteins (UCPs) has been shown to decrease ROS production (111). Moreover, it has been demonstrated the proton leak can be cytoprotective in several model of ischemic injury (112).

4.3.1. Role of Ca^{2+} on complex I derived ROS

There is an increasing body of evidence suggesting that Ca^{2+} in mitochondria can augment the oxidative stress. Increases of ROS production in isolated mitochondria have been reported upon activation of the PTP, despite the requisite mitochondrial uncoupling (19). It has been suggested that PTP opening (triggered by Ca^{2+}) induces a specific conformational change of complex I,

which results in an increase of H_2O_2 production when electrons are provided to complex I, and it may also inhibit the electron pathway inside complex I (113). This hypothesis finds its confirmation in studies in which an increase of ROS production upon inhibition of complex I has been demonstrated (91, 92, 114). The direct inhibitory effect of Ca^{2+} on complex I activity was observed in the presence of nitric oxide (NO); high levels of NO and calcium together, but not separately, caused irreversible inhibition of respiration supported by complex I substrates (glutamate and malate), but not succinate (the complex II substrate) (115). Supporting these observations, there is evidence that Ca^{2+} stimulates activity of nitric oxide synthase to generate NO, leading to inhibition of complex IV (116). A study on the role of peroxynitrite-induced permeability transition in isolated mitochondria revealed that Ca^{2+} exposes novel mitochondrial targets for nitration by ONOO⁻, consistent with protein conformational changes (117). Because mitochondrial complex I has a very complex composition and is the least well understood component of mitochondrial electron transport chain, it remains unknown if Ca^{2+} indeed leads to conformational changes. It has been reported that Ca^{2+} can alter the spectrum of cytochromes a/a₃ in isolated complex IV (118). Therefore, it is plausible that Ca^{2+} may lead to changes in complex I conformation.

4.4. ROS production at complex III

Another significant source of ROS in mitochondria is the ubisemiquinone radical intermediate (QH[•]) formed during Q cycle at the Q₀ site of complex III (119). The rate of ROS production is accelerated by inhibition of sites within complex III distal to Q₀ (the site of QH[•] formation) and further down the respiratory chain (i.e. at level of complex IV) (120). Two parameters that regulate ROS generation derived at complex III have been proposed (12). The first is dependent on the concentration of QH[•] in the Q₀ site which is increased when the distal respiratory chain is inhibited. The second involves the frequency of QH[•] occurrence which is increased when the respiratory chain turns over more quickly. Stimulation by Ca^{2+} of the TCA cycle and ox-phos enhance ROS production, at the level of respiratory chain complexes, by increasing the respiration rate and increasing the concentration of reduced substrates (121). Indeed, it has been shown that ROS generation correlates with metabolic rate (122, 123).

4.4.1. Role of Ca^{2+} on complex III derived ROS

It is not known whether Ca^{2+} can directly influence ROS production at complex III, but it is likely that Ca^{2+} can indirectly induce ROS production. Ca^{2+} can stimulate nitric oxide synthase to produce NO (116) which has been shown to inhibit complex IV. Hence, augmented radical generation at Q₀ forms a link between Ca^{2+} and ROS production at complex III. Furthermore, upon activation of PTP by Ca^{2+} , inhibition of complex III may occur due to dislocation and loss of cytochrome *c*. This can result from efflux of cytochrome *c* through an open pore or by Ca^{2+} competing with cytochrome *c* for cardiolipin binding sites, which subsequently disrupts electron transport and results in increased ROS generation (124).

Finally, as was indicated earlier, stimulation by Ca^{2+} of the TCA cycle and ox-phos enhance ROS production, at the level of respiratory chain complexes, by increasing the respiration rate and increasing the concentration of reduced substrates (121).

The study on topology of O_2^- production from different sites in the mitochondrial electron transport chain has revealed that ROS produced at complex I are released on the matrix side of the inner membrane, whereas production centered at Q_0 of complex III liberates ROS to the IMS (119). Because O_2^- cannot cross membrane, there is evidence suggesting the role of voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane in liberating anionic O_2^- from the IMS into the cytosol where it can act as a signaling molecule (125). Furthermore, Ca^{2+} ions stimulate activation of VDAC (126). Alternatively, O_2^- may be protonated to form the perhydroxyl radical which is membrane permeable (127).

4.5. Other sources of mitochondrial ROS

The respiratory chain complexes are not the only sources of mitochondrial ROS generation. Non-complex I matrix dehydrogenases are involved in ROS production in the absence of mitochondrial respiratory chain inhibitors. Reduced flavins and flavoproteins have been demonstrated to generate O_2^- (128). Purified lactate dehydrogenase (129) and glyceraldehyde-3-phosphate dehydrogenase (130) were shown to catalyze NADH-dependent O_2^- production. Flavin-dependent O_2^- production in the absence of an electron acceptor has been demonstrated for isolated mitochondrial succinate dehydrogenase (131). Among the NAD^+ -linked dehydrogenases that generated ROS, the α -ketoglutarate dehydrogenase complex (KGDHC) plays a specific role in Ca^{2+} -induced mitochondrial ROS generation. Mammalian KGDHC is composed of several copies of three enzymes: α -ketoglutarate dehydrogenase (E1; EC 1.2.4.2), dihydrolipoamide succinyltransferase (E2; EC 2.3.1.61) and dihydrolipoamide dehydrogenase (E3 or Dld; EC 1.8.1.4). Dld is also a part of other multienzyme complexes such as the pyruvate dehydrogenase complex (PDHC), ketoacid dehydrogenase complex and glycine cleavage system (132). The KGDHC is a mitochondrial enzyme tightly bound to the matrix side of the inner mitochondrial membrane and forms part of the TCA cycle enzyme super-complex (133). The fact that KGDHC is responsible for the majority of ROS production (among other non respiratory chain dehydrogenases) under maximal ADP induced respiration (state 3) or uncoupling (134), makes this enzyme an attractive focus of investigation. This is nicely illustrated by the recent discovery in yeast that dihydrolipoamide dehydrogenases are a major source of ROS (135). Moreover, it has been demonstrated that Ca^{2+} activates ROS generation by isolated KGDHC (136), as well as in other well known $[\text{Ca}^{2+}]_m$ regulated citric TCA cycle enzymes: isocitrate dehydrogenase and alpha-ketoglutarate dehydrogenase, as well as pyruvate dehydrogenase (121). It seems very plausible that increased ROS production induced by Ca^{2+} may be also a result of increased activity of KGDHC. Conditions promoting KGDHC-mediated ROS production may be mostly related to the increases in the intra-

mitochondrial NADH/ NAD^+ ratio (136). On the other hand, KGDHC is inhibited by H_2O_2 (137), and because providing NADH is the rate-limiting step of TCA cycle, KGDHC inhibition would result in decreased complex I function (138). It has also been demonstrated that H_2O_2 can impair function of other enzymes in the mitochondrial matrix such as succinate dehydrogenase (SDH) and aconitase (137). In contrast to KGDHC and SDH, aconitase activity is impaired due to direct oxidation by H_2O_2 . Because aconitase contains iron-sulfur cluster ($(4\text{Fe-4S})^{2+}$), the interaction with H_2O_2 leads to oxidation of the cluster to inactive $(3\text{Fe-4S})^{1+}$ aconitase, which is accompanied with the release of iron III from $(4\text{Fe-4S})^{2+}$ and H_2O_2 . This sequence of events leads to formation of hydroxyl radical ($\cdot\text{OH}$) by the Fenton reaction, which can exacerbate mitochondrial oxidative stress (139).

4.6. Does Ca^{2+} induce or decrease ROS production?

Ca^{2+} -induced ROS generation, at the level of respiratory chain complexes is dependent on respiratory rate (i.e. state 4 vs. state 3), the source of mitochondria, and the presence of inhibitors and uncouplers. Addition of Ca^{2+} to mitochondria isolated from heart, in the presence of antimycin A (complex III inhibitor) results in a significant increase in ROS generation (140). On the other hand, it has been shown that addition of Ca^{2+} to brain mitochondria in the presence of antimycin A did not induce ROS formation at complex III, but Ca^{2+} stimulated formation of ROS at the level of complex I in the presence of rotenone (141). It has been also shown, that an increase in Ca^{2+} uptake was correlated with O_2^- production by mitochondria from guinea pig and rat neonatal ventricular myocytes (142). A possibility exists that under physiological conditions, respiratory chain complexes involved in ROS production might be partially inhibited, hence the observed Ca^{2+} -induced ROS production (12).

A major unanswered question in the field of mitochondrial ROS production is whether mild uncoupling, or controlled H^+ leak through the IMM, stimulates ROS production or is a mechanism to minimize oxidative stress (143-145). There are multiple pathways for H^+ leak including a basal membrane leak (146), ANT mediated leak (147), and uncoupling protein (UCP) mediated leak (143). UCPS are hypothesized to protect the cell from excessive oxidative stress by reducing a high $\Delta\Psi_m$ and minimizing ROS production (144). This hypothesis is based on the idea that proton leak is sufficient to reduce $\Delta\Psi_m$ such that the protonmotive force is lowered to a point that increases respiration (and a subsequent reduction of O_2^- production generated by a highly reduced electron transport chain), but does not ameliorate ATP production by complex IV, the predominant H^+ channel in the IMM. Support for this hypothesis is found in data demonstrating that UCP2 and UCP3 mediated proton conductance is induced by O_2^- and oxidized fatty acids (94, 148, 149), but are otherwise inactive as uncouplers. A model presented by Brand and Esteves (model #2 in (144)) suggests that high electron flux resulting from fatty acid oxidation increases $\Delta\Psi_m$ and O_2^- production in mitochondria. Subsequent oxidation of *n*-6-polyunsaturated fatty acids by ROS leads to production of reactive alkenes that in turn activate proton conductance in

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UCPs, thus forming a feedback loop to control ROS production. Along these lines, Ca^{2+} influx, which consumes $\Delta\Psi_m$, should result in a partial uncoupling that would reduce reverse electron flow from Complex II and decrease ROS generation.

A fundamentally different model has been proposed in which uncoupling leads to increased ROS generation by virtue of increased electron flux (to restore and maintain $\Delta\Psi_m$), thus increasing the probability of electron slippage and an increased rate of O_2^- production at complex III (140). However, one caveat to this observation is that the complex III inhibitor antimycin A was used, thus blocking oxidative phosphorylation. In this model, Ca^{2+} can alter ROS production by simply increasing metabolic rate via TCA cycle stimulation, subsequently increasing electron flux through the electron transport chain when ox-phos is partially inhibited. In fact, increased metabolic rate has been shown to increase ROS production (150). Moreover, Ca^{2+} can activate NOS and generate NO which has been shown to inhibit complex IV, which in turn can lead to ROS production at the Q_o site of complex III (151). In support of a Ca^{2+} induced ROS generation model is data demonstrating that a mildly uncoupling drop of less than 10 mV in $\Delta\Psi_m$ induced by low concentrations of the protonophore FCCP in cerebellar granule neurons led to an increase in oxidative stress and ultimate loss of Complex V activity and ATP starvation (152). Experiments on UCP (n) mediated uncoupling are made even more difficult by the fact that UCP2 and UCP3 are found at a tiny relative abundance of 1% or less than that of the well characterized UCP1 in brown adipose tissue mitochondria (153). Thus, Ca^{2+} may both stimulate ox-phos electron flux, as well as leading to partial inhibition of the electron transport chain, leading to an increased probability of electron slippage to O_2 .

An additional possible avenue of Ca^{2+} and ROS crosstalk involves influx of K^+ through the mitochondrial K_{ATP} channel (mito K_{ATP}) which has been suggested induce an increase in mitochondrial ROS generation. Andrukhiv *et al.* recently demonstrated that mito K_{ATP} activation (or low concentrations of the K^+ ionophore valinomycin) increase mitochondrial ROS generation in isolated rat cardiac mitochondria despite an increase in respiratory electron flux (154). Similar results were seen upon K^+ influx via opening of the mitochondrial Ca^{2+} sensitive K^+ channel (mtBK $_{\text{Ca}^{2+}}$) (155). Garlid's group showed that mitochondrial K^+ influx leads to matrix alkalinization as H^+ ions pumped out via the respiratory chain are replaced by K^+ ions (156). Moreover, it was determined that matrix alkalinization leads to inhibition of electron transport at complex I (154) supporting data that matrix alkalinization leads to increased mitochondrial ROS production (157, 158). Therefore, while slight uncoupling due to electrophoretic K^+ influx leads to a decrease in $\Delta\Psi_m$, a secondary effect of complex I inhibition is matrix alkalinization which stimulates ROS production. Furthermore, PKC ϵ , which is a protein kinase present in cardiac mitochondria (159) is an activator of mito K_{ATP} (160). PKC ϵ is activated by PKG (160), which is in turn stimulated by Ca^{2+} via the NO/cGMP/PKG signaling

pathway, linking Ca^{2+} to mito K_{ATP} mediated ROS production. However, as discussed above, these results are conflict with studies that demonstrate that uncoupling lead to a decrease in ROS generation (161, 162). In fact, a number of studies from Kowaltowski's group have demonstrated that mito K_{ATP} opening reduces ROS generation (163-165). Ferranti *et al.* showed that the mito K_{ATP} opener diazoxide (DZX) leads to a decrease in H_2O_2 released from isolated rat heart, brain and liver mitochondria using the ROS indicator Amplex Red (165). These conflicting data have been suggested to result from direct interactions between DZX and commonly used ROS probes such as dichlorofluorescein, or by changes in indicator fluorescence by pH changes induced by $\Delta\Psi_m$ dependant probe uptake (164, 166). Despite these conflicting data, mitochondrial K^+ flux appears to affect mitochondrial ROS generation.

5. ROS AND Ca^{2+} SIGNALING

Supported by an expansive library of literature describing the pathological effects of ROS and oxidative damage, a developing field of study has initiated investigations on ROS as a physiological mediator of normal cellular function (167). As described above, mitochondria are crucial to proper cellular Ca^{2+} signaling and energy production, and as a consequence, are a significant source of cellular ROS. Just as Ca^{2+} plays a role in the production of ROS, cellular redox state can significantly modulate Ca^{2+} signaling (for review see, (103, 168-172)). The reciprocal interaction between Ca^{2+} modulated ROS production and ROS modulated Ca^{2+} signaling underlies the concept of ROS and Ca^{2+} crosstalk. This section will discuss how redox state and ROS modulate Ca^{2+} ion channels and pumps and the intricate crosstalk between ROS and Ca^{2+} signaling.

5.1. Redox modulation of Ca^{2+} transporters

Many cellular processes are modulated by the redox state of proteins. Changes in structure caused by oxidizing neighboring cysteine residues (resulting in disulfide bridge formation) can change the activity of enzymes and ion transporters. In the context of Ca^{2+} signaling, redox state and ROS can stimulate as well as inhibit Ca^{2+} channels/pumps/exchangers, generally increasing Ca^{2+} channel activity and inhibiting Ca^{2+} pumps. Regulation is found intracellularly at cellular store Ca^{2+} channels and at PM channels. In the following section, we will discuss known instances of redox regulation of Ca^{2+} ion channels, pumps, and exchangers, and when observed, discuss how ROS can directly modulate those Ca^{2+} transporting proteins.

5.1.1. Ryanodine receptors (RyR) are stimulated by oxidation

Following action potential-induced depolarization of the plasma membrane, Ca^{2+} enters the cell via L-type Ca^{2+} channels, evoking Ca^{2+} -induced Ca^{2+} release (CICR) from SR Ca^{2+} stores (173). Ryanodine receptors are the primary Ca^{2+} release channel involved in SR Ca^{2+} release to evoke muscle contraction in skeletal (RyR1 isoform) and cardiac (RyR2 isoform) muscle cells.

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RyR are large (>500 kDa) proteins that form tetramers in the SR and ER membranes (174). Despite having up to 89 cysteine residues per monomer, only a small subset has been shown to be redox active and play a role in channel function (175-177). Experimentally, generation of RyR protein disulfides led to reversible activation of channel activity (13, 178-181). Moreover, shifts in the ratios of cellular redox buffers such as the GSH/GSSG redox pair (178, 182-184) and the NADH/NAD⁺ redox pair (185-187) modulate RyR channel activity. In addition to changes in RyR channel modulation by changes in whole cell redox homeostasis, ROS has been shown to directly modulate RyR (188). O_2^- (187, 189) and H_2O_2 /hydroxyl radical (190-192) directly oxidize redox-sensing thiols on the RyR leading to channel stimulation, and effect that is reversible by agents that reduce thiol groups. Moreover, ROS induction of NOS can oxidize NO to nitrosium (NO^+) and subsequently react with free RyR thiols to form S-nitrosothiol which stimulates RyR channel activity (175, 193).

5.1.2. IP₃R are stimulated by oxidation

Inositol 1,4,5-triphosphate receptors (IP₃R) channels are the primary Ca^{2+} release channel in the endoplasmic reticulum (ER) in non-excitabile cells and constitute a minor proportion of SR Ca^{2+} release channels in cardiac cells (194). ER Ca^{2+} release via IP₃R is initiated by binding of the signaling molecule inositol 1,4,5-triphosphate (IP₃). In non-excitabile cells such as HeLa and the cerebellum, redox modification of sulfhydryls is thought to be responsible for a thimerosal dependant sensitization to IP₃ (195). GSSG has also been shown to stimulate IP₃R channel activity by increasing the binding affinity of IP₃ to hepatocyte IP₃R (196, 197). ROS has been shown to directly stimulate IP₃R mediated Ca^{2+} release from the ER. ROS generated by tert-butyl hydroperoxide treatment of hepatocytes resulted in DTT reversible stimulation of IP₃R Ca^{2+} release (198). H_2O_2 treatment resulted in Ca^{2+} release from endothelial cells (199). Xanthine/Xanthine oxidase generated O_2^- was shown to stimulate IP₃R channel activity in smooth muscle cells (200).

5.1.3. SERCA and PMCA are inhibited by oxidation

Muscle relaxation following Ca^{2+} release from SR/ER Ca^{2+} stores is facilitated by Ca^{2+} re-uptake by the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA). SERCA activity is sensitive to the redox state, but unlike RyR and IP₃R, SERCA is inhibited by oxidation and ROS (201). There are three SERCA isoforms expressed differentially in different tissue types that have up to 25 cysteine residues with one or two essential catalytic cysteines (202). Thiol oxidizing compounds inhibit SERCA Ca^{2+} pumping activity while reducing agents including DTT and GSH stimulate SERCA (203). ROS have a striking effect on SERCA on two levels. Oxygen radicals have been shown to depress SERCA in cardiac cells (189, 204-206) and nearly inhibit SERCA in smooth muscle cells (207, 208). SERCA is dependent on ATP hydrolysis to energize Ca^{2+} pumping. H_2O_2 and O_2^- have also been shown to directly inhibit ATP binding to SERCA, uncoupling ATP hydrolysis from Ca^{2+} pumping (188, 189, 209, 210).

The plasma membrane Ca^{2+} ATPase (PMCA) is a much slower pump than SERCA. Although $\text{Na}^+/\text{Ca}^{2+}$ exchange is the dominated efflux mechanism, PMCA works alongside with $\text{Na}^+/\text{Ca}^{2+}$ exchange for maintenance of low intracellular Ca^{2+} during the relaxation phase of muscle contraction (211). As with the SERCA, ROS have been shown to modify PMCA sulfhydryls, depressing activity as well as inhibiting ATP hydrolysis, thus reducing Ca^{2+} pumping in the heart (206, 212), pancreas (213), and brain (214).

5.1.4. Plasma membrane $\text{Na}^+/\text{Ca}^{2+}$ exchanger is modulated by oxidation

The Plasma membrane $\text{Na}^+/\text{Ca}^{2+}$ exchange (NCX) is a high capacity, low affinity Ca^{2+} pump that utilizes the transmembrane Na^+ gradient to exchange extracellular Na^+ for cellular Ca^{2+} in a ratio of 3:1. There is evidence suggesting ROS both stimulate and decrease NCX activity. In cardiac cells, ROS in the form of both H_2O_2 (215) and O_2^- (216) was shown to stimulate NCX activity. In another study, H_2O_2 stimulated NCX while the strong oxidant HOCl led to NCX inhibition. H_2O_2 was not found to augment NCX activity when expressed in CHO-K1 cells, but peroxynitrite and peroxy radical generation led to a decrease in NCX activity (217, 218). During oxidative stress, either stimulation or inhibition of NCX could lead to Ca^{2+} dysregulation and have pathological effects. It is known that extended membrane depolarization or high intracellular Na^+ can lead to NCX operating in reverse, ultimately acting as a Ca^{2+} importer

6. CROSSTALK BETWEEN Ca^{2+} AND ROS

As illustrated above, there is clearly an intricate level of crosstalk between Ca^{2+} signaling in the cell and the redox environment, which in turn is modulated to a large extent by both physiological and pathological generation of ROS. In mitochondria, Ca^{2+} signals are central to metabolic regulation of the TCA cycle and ox-phos of which ROS generation is an important by-product. Most ROS are buffered and detoxified very quickly by mitochondrial and cellular antioxidant defenses (SOD, catalase, GSH for example), so the extent of ROS modulation of Ca^{2+} signaling is most likely dependent on spatiotemporal positioning within microenvironments of transiently higher oxidative potential. There is considerable data supporting Ca^{2+} microdomains (219-221), yet relatively little discussion of redox microdomains. The following section will describe the features of mitochondrial Ca^{2+} microdomains, followed by discussion of crosstalk with ROS microdomains and localized regions of differential redox potential.

6.1. Mitochondrial Ca^{2+} microdomains

There are many types of subcellular Ca^{2+} release events that occur as a result of different species and numbers of Ca^{2+} channels opening (222). The physiological basis of Ca^{2+} microdomains depends on a localized concentration of Ca^{2+} around the mouth of an open Ca^{2+} release channel that is significantly above that of the steady state $[\text{Ca}^{2+}]_c$. For instance, it has been estimated that $[\text{Ca}^{2+}]_c$ can reach 100 μM within 20 nm of open plasma

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membrane Ca^{2+} channels (223). Moreover, measurements of $[\text{Ca}^{2+}]_c$ formed by localized ER Ca^{2+} release (sparks) has been estimated to be between 20 and 100 μM (224-227). These loci of $[\text{Ca}^{2+}]_c$ orders of magnitude higher than resting $[\text{Ca}^{2+}]_c$ have profound effects on mitochondrial Ca^{2+} uptake. Recent data characterizing the Ca^{2+} affinity of the MCU calculated the half-saturation of current to be 19 +/- 2 mM Ca^{2+} (24), which is much higher than previous estimates of between 1 and 189 μM (53), indicating the MCU is most effective at high $[\text{Ca}^{2+}]_c$. In contrast, resting cytosolic $[\text{Ca}^{2+}]_c$ has been estimated to be < 100-300 nM (228, 229). Therefore, mitochondrial Ca^{2+} uptake by the MCU would be most effective when $[\text{Ca}^{2+}]_c$ reach tens to hundreds of μM , as occurs in the direct vicinity of SR/ER Ca^{2+} release channels upon activation.

For mitochondrial Ca^{2+} microdomains to serve a physiological role, there must be a close apposition between ER/SR Ca^{2+} release channels and mitochondrial Ca^{2+} uptake channels to take advantage of the high localized $[\text{Ca}^{2+}]_c$. This has been shown to be true immunologically as well as via direct visualization. In HeLa cells, a large fraction of mitochondria lie within 100 nm of the ER as determined by co-localization of fluorescence from GFPs localized to the ER lumina and mitochondrial matrix (230). IP₃R are enriched in regions apposed to mitochondria as determined via increased IP₃R immunoreactivity near mitochondria in astrocytes and oligodendrocytes (231, 232) and by direct electron microscopy in Purkinje neurons (233, 234). Via electron microscopy, it was demonstrated that the distance between SR RyR and the nearest mitochondrial surface is very short (between 37 and 270 nm) in cardiac ventricular myocytes (235). In fact, peptide linkages have been observed in that may help to position mitochondria near calcium release channels, leading to a distance between ER and mitochondria of between 5-30 nm at the junctions (236). Privileged Ca^{2+} exchange between the ER/SR and mitochondria may be further enhanced if mitochondrial Ca^{2+} uptake channels are enriched in the vicinity of corresponding ER/SR Ca^{2+} release channels. However, until the molecular identity of the MCU can be established, this facet of microdomain Ca^{2+} transfer remains unclear.

6.2. Mitochondrial ROS microdomains

In part because the technology to spatially and temporally visualize ROS currently lacks the resolution found with modern Ca^{2+} visualization tools, the concept of redox or ROS microdomains has not received much attention. Nonetheless, some of the fundamental properties of Ca^{2+} microdomains can be extended to ROS signaling. As described above, mitochondria are the primary source of cellular ROS. By virtue of cellular architecture, mitochondria define a localized source of ROS, similar to the spatially defined Ca^{2+} release mechanisms that define Ca^{2+} microdomains. Moreover, the action of ROS buffering and defense mechanisms ensure the lifetime of ROS is fairly short within the cell, facilitating temporal fluctuations by eliminating ROS pulses. Redox microdomains differ from Ca^{2+} microdomains however, in that changes in the redox environment are generally formed by a steady state change in ROS production or decrease in

ROS defense rather than a transient release of bulk ROS. Thus, a redox microdomain may be spatially defined, but persist as a concentration gradient rather than as a pulse. Additionally, ROS microdomains result from the combined redox effects of different species with differing diffusion abilities including membrane permeable H_2O_2 , membrane impermeable O_2^- , as well as changes in the ratios of redox couples such as GSH/GSSG and the local availability of defensive enzymes. To summarize, ROS microdomains are generally formed by diffusion from spatially defined sources rather than by release from a mitochondrial store of ROS. However, the recent discovery of an ROS activated inner mitochondrial anion channel (IMAC) (237) provides the possibility of transient ROS pulses, similar to the well known phenomena of Ca^{2+} sparks (238).

In cardiac mitochondria loaded with TMRM (a $\Delta\Psi_m$ sensitive fluorescent probe), a local loss $\Delta\Psi_m$ was detected upon application of a laser flash resulting in the production of O_2^- and OH by photoexcitation (239). Laser de-energized mitochondria transiently produced ROS in two distinct phases; an initial slow rise termed “trigger ROS” caused by accumulation of photoexcitation-related ROS, followed by a “ROS burst” that accrued simultaneously with transient mitochondrial membrane depolarization. This phenomenon, called “ROS-induced-ROS release” (RIRR), was initially postulated to be dependent on the PTP for propagation of ROS in mitochondria (239). Further studies have shown that the inner mitochondrial anion channel (IMAC) plays a crucial role in propagation of ROS in RIRR. The IMAC channel is itself activated by a ROS dependent reduction in GSH:GSSG ratio, indicative of a change in mitochondrial protein thiol status, and opens prior to PTP opening (237, 240, 241). In a model presented by Aon *et. al.* (237), recycling of oxidized glutathione to restore a high GSH:GSSG ratio is carried out by glutathione reductase, consuming NADPH in the process. NADPH is subsequently regenerated by NADH transhydrogenase which has been suggested to involve partial uncoupling via UCP2 (242). Thus, in the face of enhanced ROS production, NADH is shunted to restore the GSH:GSSG ratio in order to maintain redox homeostasis at the expense of ox-phos reducing power (NADH supply) and a diminution of $\Delta\Psi_m$. The extent to which Ca^{2+} regulation in this process is currently unknown.

6.3. Mitochondrial Ca^{2+} and ROS crosstalk

As a central player in cellular Ca^{2+} signaling, energy metabolism, and apoptosis, it is essential that mitochondria have the capacity to micromanage Ca^{2+} handling. As such, it not surprising that ROS produced as a by-product of ox-phos is utilized as a signaling molecule to fine tune Ca^{2+} transport, both at the mitochondrial level, as well as the ER/SR. The sheer number of diseases and disorders that are caused by Ca^{2+} deregulation and/or oxidative stress emphasize the importance of tight regulation in these pathways.

Figure 3 illustrates how a Ca^{2+} signal initiated during cardiomyocyte excitation-contraction can lead to changes in both Ca^{2+} handling, as well as ROS generation.

Crosstalk signaling between mitochondrial Ca^{2+} and ROS

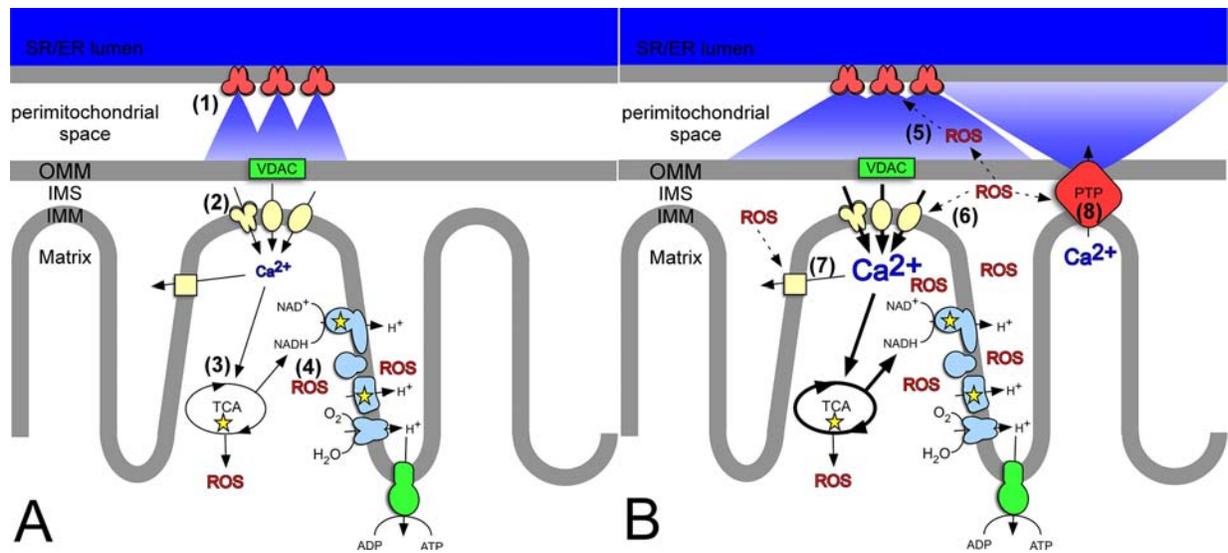


Figure 3. Scheme for Ca^{2+} /ROS crosstalk in cardiac mitochondrial microdomains. **A:** Ca^{2+} stimulation of ox-phos and ROS production. Pulses of Ca^{2+} resulting from excitation-contraction derived SR RyR Ca^{2+} release (in red, (1)) leads to mitochondrial Ca^{2+} uptake via MCU, RaM, and mRyR (in yellow, (2)). Increased $[\text{Ca}^{2+}]_m$ stimulates TCA cycle enzymes (3) which generates NADH that feeds into the respiratory electron transport chain (in blue), in turn increasing APT synthesis and ROS production (4). **B:** ROS modulation of Ca^{2+} channel activity. Diffusion of ox-phos derived ROS eads to a shift in redox homeostasis resulting in a locally oxidizing environment. Redox and ROS modulation of Ca^{2+} channels increases Ca^{2+} release from SR RyR channels (5), increased mitochondrial Ca^{2+} uptake by mitochondrial Ca^{2+} channels (6), and changes in $\text{Na}^+/\text{Ca}^{2+}$ exchange (7). Continued Ca^{2+} uptake can lead to Ca^{2+} overload and further Ca^{2+} induced ROS generation which can ultimately lead to PTP opening (8) and mitochondrial dysfunction. IMM, inner mitochondrial membrane; IMS, intermembrane space; OMM, outer mitochondrial membrane; SR/ER, sarcoplasmic reticulum/endoplasmic reticulum; VDAC, voltage dependant anion channel; PTP, permeability transition pore.

Briefly, an excitation-contraction derived Ca^{2+} transient generated by SR-RyR channel opening results in mitochondrial Ca^{2+} uptake. Increased mitochondrial Ca^{2+} stimulates metabolic pathways leading to an increase in reduced substrates and electron transport which can evoke subsequent increases in ROS generation by the mechanisms detailed above. ROS can then stimulate further Ca^{2+} release by oxidizing SR-RyR cysteine residues. For example, it has been shown that mitochondrially derived ROS can elicit cerebral artery dilation by activating RyR Ca^{2+} sparks (243). Moreover, there is some evidence that mitochondrial Ca^{2+} transport is under redox control as well (our results, unpublished and (244)), although the mechanisms are not known. In this model, a mitochondrial Ca^{2+} signal is utilized to match energy production via ox-phos to the energy demands of muscle contraction. A slight increase in ROS may sensitize the Ca^{2+} signaling pathways during times of rapid signaling or high energy demand. Furthermore, this crosstalk is made possible by virtue of microdomain architecture. Based on similarities to neuronal structures, microdomain Ca^{2+} signaling has been described as being “quasi-synaptic” between SR and mitochondria (225). The comparison was conceptually extended by Camello’s group to include ROS as a “neuromodulator” (170). Another instance in which ROS may affect Ca^{2+} signaling is illustrated by Ca^{2+} oscillations in HeLa cells. Ca^{2+} oscillation were shown to be invoked upon release of mitochondrial Ca^{2+} via $\text{Na}^+/\text{Ca}^{2+}$ exchange near ER-IP₃R, invoking secondary Ca^{2+} induced release

from the ER through IP₃R (245). Oscillations caused by IP₃R sensitization by low levels of mitochondrially generated ROS in Ca^{2+} treated cells were subsequently abolished by antioxidants.

The positive stimulation of mitochondrial Ca^{2+} signals by ROS and increased ROS generation resulting from increased $[\text{Ca}^{2+}]_m$ can lead to a positive feedback loop. While physiological increases in $[\text{Ca}^{2+}]_m$ are beneficial for metabolism, overload is detrimental to mitochondrial function and can become pathological. Likewise, ROS as a signal transduction molecule is physiologically relevant, but excess ROS leads to oxidative stress and mitochondrial dysfunction. Following short periods of excitation and enhanced Ca^{2+} signaling, mitochondrial antioxidant defenses should restore ROS to physiological levels and mitochondrial Ca^{2+} efflux channels should expel excess Ca^{2+} . However, during prolonged stimulation or in the face of oxidative stress, there must be mechanisms to break the feedback cycle to prevent Ca^{2+} overload. As mentioned above, there is evidence that transient PTP flicker can act as a “pressure relief valve”, expelling excess Ca^{2+} . One problem with this hypothesis is that a full opening of PTP would lead to the release of GSH, diminishing the antioxidant capacity of the mitochondria. Therefore, the existence of a “sub-conductance” state of PTP must be proposed. Additionally, there is evidence that ROS activation of c-Jun N-terminal kinase can lead to an up-regulation of glycogen synthase.

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Increased glycogen synthase activity effectively shunts glucose metabolism away from pyruvate production. Lower pyruvate levels subsequently translate into a reduction of mitochondrial substrate and a reduction in metabolism, reducing ROS production (246). A third possibility is that mitochondrial Ca²⁺ efflux mechanisms such as Na⁺/Ca²⁺ are also modulated by ROS, thereby resulting in an increase of total Ca²⁺ flux, both in and out of the mitochondria.

7. PERSPECTIVES

Despite great advances in mitochondrial Ca²⁺ dynamics and ROS metabolism and signaling, there are clearly more questions than answers in this field. From what was once believed to be a dangerous waste product of respiration, ROSs have only recently come to the forefront as important signaling molecules. Moreover, the idea that ROS may play a required role in modulating Ca²⁺ signaling has been a startling discovery. It is becoming increasingly important to address the mechanisms by which mitochondrial Ca²⁺ regulate mitochondrial ROS generation. There is considerable evidence that ROS and the redox environment modulate cellular Ca²⁺ channels. However, our knowledge of how ROS and the redox environment modulate mitochondrial Ca²⁺ transport is lacking. Perhaps one of the biggest hurdles in this field is that no mitochondrial inner membrane ion channel has ever been cloned. Furthermore, specific ROS probes targeted to the mitochondria of living cells are still underdeveloped. Clearly, the field of mitochondrial microdomains and the crosstalk between Ca²⁺ and ROS represents a new frontier in biomedical research. Once we understand the dynamic interplay between these two crucial signaling pathways we will be poised to develop therapies for the large number of insidious diseases involving mitochondrial dysfunction.

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Abbreviations: ACON: aconitase, ADP: adenosine diphosphate, α -KGDH: α -ketoglutarate dehydrogenase, ANT: adenine nucleotide transporter, ATP: adenosine triphosphate, [Ca²⁺]_c: cytoplasmic calcium concentration, [Ca²⁺]_m: mitochondrial calcium concentration, CAT: catalase, CICR: calcium-induced calcium release, CS: citrate synthase, Cu/ZnSOD: copper/zinc superoxide dismutase, ER: endoplasmic reticulum, ETC: electron transport chain, FADH: reduced flavin adenine dinucleotide, FUM: fumarase, GSH: glutathione, GSSG: glutathione disulfide, H₂O₂: hydrogen peroxide, ICDH: isocitrate dehydrogenase, IMAC: inner membrane ion channel, IMM: inner mitochondrial membrane, IMS: intermembrane space, IP₃R: inositol-1,4,5-triphosphate receptor, MCU: mitochondrial calcium uniporter, MDH: malate dehydrogenase, MnSOD: manganese superoxide dismutase, MPT: mitochondrial permeability transition, mRrR: mitochondrial ryanodine receptor, NADH: reduced nicotinamide adenine dinucleotide, NADPH: reduced nicotinamide adenine dinucleotide phosphate, O₂⁻: superoxide, OMM: outer mitochondrial membrane, ox-phos: oxidative phosphorylation, PDH: pyruvate dehydrogenase, PTP: permeability transition pore, RAM:

rapid mode, RIRR: ROS-induced ROS release, ROS: reactive oxygen species, RuR: ruthenium red, SDH: succinate dehydrogenase, SR: sarcoplasmic reticulum, TCA: tricarboxylic acid cycle, THD: NADH transhydrogenase, TMRM: tetramethylrhodamine methyl ester, TPx: thioredoxin peroxidase, TR: thioredoxin reductase, TR^{ox}: oxidized thioredoxin, TR^{red}: reduced thioredoxin, VDAC: voltage dependant anion channel, $\Delta\Psi_m$: membrane potential

Key words: Calcium, Mitochondria, Reactive Oxygen Species, Crosstalk, Sarcoplasmic Reticulum, Redox, Microdomain, Mitochondrial Permeability Transition, Permeability Transition Pore, Apoptosis, Ryanodine Receptor, Review

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