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Mitochondrial Ion Channels/Transporters as Sensors and Regulators of Cellular Redox Signaling

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Abstract

Significance: Mitochondrial ion channels/transporters and the electron transport chain (ETC) serve as key sensors and regulators for cellular redox signaling, the production of reactive oxygen species (ROS) and nitrogen species (RNS) in mitochondria, and balancing cell survival and death. Although the functional and pharmacological characteristics of mitochondrial ion transport mechanisms have been extensively studied for several decades, the majority of the molecular identities that are responsible for these channels/transporters have remained a mystery until very recently. **Recent Advances:** Recent breakthrough studies uncovered the molecular identities of the diverse array of major mitochondrial ion channels/transporters, including the mitochondrial Ca^{2+} uniporter pore, mitochondrial permeability transition pore, and mitochondrial ATP-sensitive K^+ channel. This new information enables us to form detailed molecular and functional characterizations of mitochondrial ion channels/transporters and their roles in mitochondrial redox signaling. **Critical Issues:** Redox-mediated post-translational modifications of mitochondrial ion channels/transporters and ETC serve as key mechanisms for the spatiotemporal control of mitochondrial ROS/RNS generation. **Future Directions:** Identification of detailed molecular mechanisms for redox-mediated regulation of mitochondrial ion channels will enable us to find novel therapeutic targets for many diseases that are associated with cellular redox signaling and mitochondrial ion channels/transporters. *Antioxid. Redox Signal.* 21, 987–1006.

Introduction

OVER THE YEARS, redox signaling has been recognized as an important system for cellular functions in all cell types/tissues (44, 135, 155, 177, 182). Cells maintain redox balance through generation and breakdown of reactive oxygen species (ROS) and nitrogen species (RNS). High levels of ROS and RNS are known to promote cell damage and death. However, recent evidence indicates that the production of low to moderate levels of ROS/RNS is also critical for proper regulation of many essential cellular processes (53, 155, 158, 175, 177, 182). Redox signaling has also gained recognition for its role in mediating diverse tissue-specific cellular functions across a wide range of cell types. For instance, redox signaling regulates muscle contraction/relaxation (145, 155, 169, 178),

insulin secretion from pancreatic beta cells (191), metabolic cycles in liver (137), self-renewal and differentiation in stem cells (98), and T-cell homeostasis (67, 100, 120).

Since Szent-Gyorgyi first provided the initial concept in 1967 regarding a possible role for electronic mobility in biological materials as one of the fundamental mechanisms for cellular signaling (170), and the area of redox biology has witnessed a burst of major remarkable discoveries. These include (i) free radical superoxide (O_2^-) production by cellular enzymes (112), (ii) superoxide dismutase that can catalytically scavenge O_2^- , blunt the cascade of oxidation, and neutralize oxygen toxicity (113), and (iii) a membrane-bound enzyme complex, nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase (11). Since free radicals are highly reactive with cellular lipids, DNA, and proteins and can bring about

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harmful oxidations, research in the early years concentrated on the chemistry of the individual ROS and the cellular damage that they bring about.

From the late 1970s to the early 1980s, there were several milestone discoveries, which brought the new concept that redox also serves as a signaling molecule to maintain cellular functions. Oberley and Buettner proposed that lower concentrations of O_2^- or hydrogen peroxide (H_2O_2) induce cell division (125). Mukherjee *et al.* showed that H_2O_2 is involved in the physiological response to insulin in adipocytes (119). These reports first showed that ROS were not simply involved in cellular toxicity. Another important finding is nitric oxide (NO) and its biological action on vascular smooth muscles. Mittal and Murad's group found that NO was capable of activating guanylate cyclase and generating cyclic GMP (10). This critical observation was later followed by the important finding of the identification of endothelial-derived relaxing factor as NO by Ignarro and Kadowitz' group (76–78) and Moncada's group (133). From these discoveries, redox biology has finally broadened the field into signal transduction research and has drawn major attention from both general biologists and the medical research community.

O_2^- and NO are the primary ROS and RNS, respectively, that are produced in cells under physiological and pathophysiological conditions, which react with other molecules (and each other) to form a diverse form milieu of additional ROS and RNS such as H_2O_2 and peroxynitrite ($ONOO^-$) (177, 182). Unlike most other endogenous second messengers for signal transduction, H_2O_2 and NO are highly active molecules that readily diffuse across cell membranes and cannot be stored inside cellular compartments/organelles. Therefore, their signaling capacity should be tightly controlled at the levels of their local biosynthesis and availability. Indeed, subcellular ROS/RNS concentration is regulated by a diverse subset of enzymes that are localized in each cellular compartment which contributes to their local generation and breakdown. Notably, mitochondria are the most important cellular compartment/organelle for tuning the cellular ROS/RNS concentration, as they contain various ROS/RNS generation systems as well as detoxification enzymes. The electron transport chain (ETC) located in the inner mitochondrial membrane (IMM) is the main cellular source of ROS (Fig. 1). There are a number of studies that suggest the presence of an NO synthase (NOS) in mitochondria (mtNOS), which produces NO in mitochondrial matrix (Fig. 1) (58, 194). However, the existence/function of mtNOS is highly controversial (28, 194) (see Overview: Mitochondrial Ion Channels/Transporters, ETC, and Mitochondrial Redox Signaling and Regulation of ETC Activity by Mitochondrial NO Signaling sections).

Under physiological conditions, the balance of mitochondrial ROS/RNS homeostasis is under tight control of mitochondrial ion channels/transporters that are located in IMM and outer mitochondrial membrane (OMM) (Fig. 1). In pathophysiological conditions, intra- or extra-cellular ROS/RNS stress/signaling is transmitted to mitochondria through redox-dependent post-translational modification (PTM) of mitochondrial ion channel/transporters, amplified by mitochondrial ROS/RNS generation systems, which subsequently release excessive ROS/RNS from mitochondria into the cytosol through specific mitochondrial ion channels/transporters such as the mitochondrial permeability transition pore (mPTP) and the inner membrane anion channel (IMAC),

which triggers ROS-induced ROS release in neighboring mitochondria (26, 197) and finally, activates cell-death signaling (38) (Fig. 1). Therefore, mitochondrial ion channels/transporters serve as critical sensors and regulators of cellular redox signaling and also in balancing cell survival and death. Especially, the redox-mediated PTMs of mitochondrial ion channels/transporters and ETC serve as key mechanisms for the regulation of mitochondrial ROS/RNS generation (see Overview: Mitochondrial Ion Channels/Transporters and Redox-Dependent PTMs section). Although the functional and pharmacological characteristics of mitochondrial ion transport mechanisms have been well studied for several decades, the majority of the molecular identities that are responsible for the mitochondrial ion channels/transporters have remained a mystery until very recently, owing to the use of updated techniques such as whole genome screening, proteomics, and genetic manipulations (132). These efforts by many researchers finally reached a diverse array of breakthroughs in identifying the coding genes responsible for mitochondrial ion channels/transporters. These include mitochondrial Ca^{2+} uniporter pore (MCU) (15, 46) and its regulatory proteins (MICU1–3 and MCUR1) (110, 139, 141), mitochondrial Na^+/Ca^{2+} exchanger (mNCE) (134), mPTP (24, 61), and mitochondrial ATP-sensitive K^+ channel (mito- K_{ATP}) (54). This new information enables us to understand more detailed molecular and functional characterizations of mitochondrial ion channels/transporters and their roles in mitochondrial redox signaling.

In this review, we describe the importance of spatiotemporal control of ROS/RNS at mitochondrial levels and their regulation by mitochondrial ion channels/transporters and ETC, especially focusing on Ca^{2+} -dependent regulation of mitochondrial redox signaling and redox-dependent PTMs. We will introduce recent progress in the molecular identification of mitochondrial ion channels/transporters. We will also discuss the proposed feedback mechanism as to how redox-dependent PTMs of mitochondrial ion channels/transporters and ETC can fine-tune mitochondrial redox signaling.

Overview: Mitochondrial Ion Channels/Transporters, ETC, and Mitochondrial Redox Signaling

As originally proposed by Mitchell and Molye, mitochondria have been well studied as cellular “powerhouse” organelles for producing ATP (92), but recently, they have been recognized as important regulators for multiple signal transduction pathways, including redox signaling. The majority of mitochondrial ATP is produced by oxidative phosphorylation (OXPHOS) through the ETC, which is a concerted series of redox reactions catalyzed by multi-subunit enzymes that are embedded in the IMM (Figs. 1 and 2). Through the slippage of an electron from the ETC to molecular oxygen during OXPHOS, O_2^- is continuously produced as a primary oxygen-free radical in mitochondria (Figs. 1 and 2). This “constitutive” O_2^- generation brings mitochondria to the center stage for cellular redox regulation in all cell types/tissues. The ETC also functions as a proton (H^+) pump to shuttle electrons within the mitochondrial intermembrane space (IMS) to build up the mitochondrial membrane potential ($\Delta\Psi_m$). Unlike other organelles, mitochondria possess unique double-membrane structures with distinct phospholipids and protein

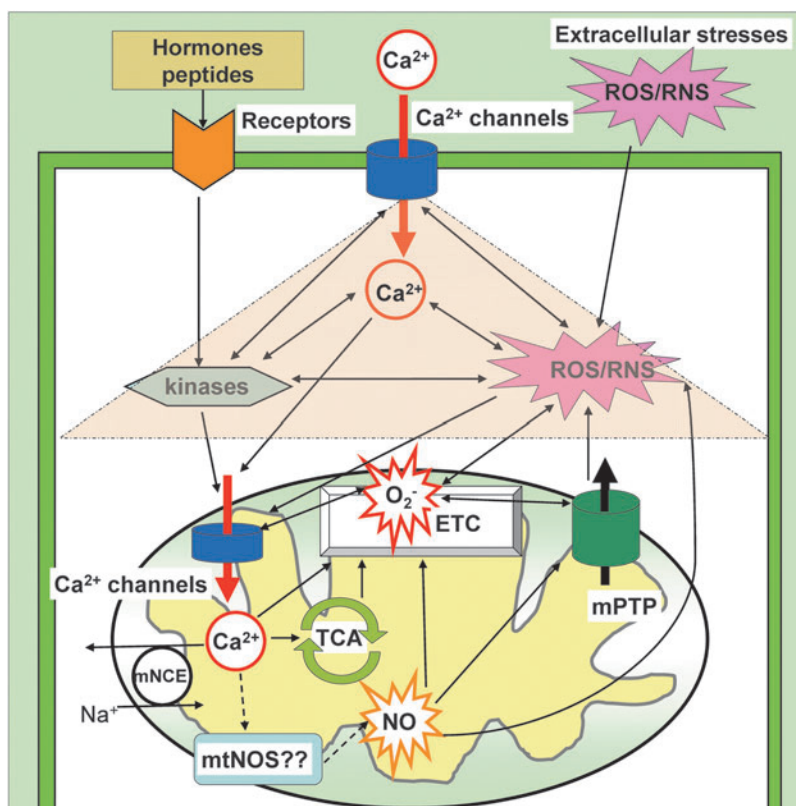


FIG. 1. Overview of mitochondrial ion channels/transporters and redox signaling. Under physiological conditions, the balance of mitochondrial ROS/RNS is tightly controlled by multiple mitochondrial ion channels/transporters that are located at the IMM and the OMM (OMM structure is abbreviated in this figure). In pathological conditions, plasma membrane receptor (orange) stimulation, extracellular Ca²⁺ elevation, and/or exogenous ROS/RNS elevation (pink burst) trigger cellular signal transduction *via* kinase cascades, cytosolic Ca²⁺ elevation, and cytosolic ROS/RNS generation. These are amplified through reciprocal action (pink triangle) and transmit into mitochondria through redox-dependent PTMs of mitochondrial ion channels/transporters, especially Ca²⁺ channels at the IMM (blue). Mitochondrial Ca²⁺ efflux is mainly regulated by an mNCE. The PTMs of mitochondrial ion channels/transporters change the activity of the Ca²⁺ influx mechanism at IMM and induce Ca²⁺ accumulation into the mitochondrial matrix, which affects the efficiency of electron flow, ATP production, and O₂⁻ generation at ETC. The produced O₂⁻ reacts with other molecules (also each other) and forms additional ROS. The existence of mtNOS and the production of NO in matrix are still controversial. The excessive ROS/RNS in mitochondria are released into the cytosol through specific mitochondrial ion channels/transporters such as the mPTP (green) and the IMAC (not shown in the figure). IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane; mNCE, mitochondrial Na⁺/Ca²⁺ exchanger; mPTP, mitochondrial permeability transition pore; IMAC, inner membrane anion channel; ETC, electron transport chain; mtNOS, mitochondrial nitric oxide synthase; O₂⁻, superoxide; PTM, post-translational modification; ROS, reactive oxygen species; RNS, reactive nitrogen species. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

compositions, which enable mitochondria membranes to maintain $\Delta\Psi_m$ (39, 92). Generally, to minimize the energy loss, the ion permeability of the IMM is kept low, but the IMM contains a variety of ion channels/transporters (128, 129, 132, 138) that regulate the efficiency of redox reactions at the ETC (29, 129). As summarized in Figure 1, mitochondrial ion channels/transporters receive cell signaling information from the cytosolic side through (i) cytosolic Ca²⁺ elevation, (ii) phosphorylation by Ca²⁺- and/or redox-dependent kinases, and (iii) cytosolic ROS/RNS-dependent PTMs (see also Overview: Mitochondrial Ion Channels/Transporters and Redox-Dependent PTMs section) (Fig. 1). These three elements can receive extracellular signals such as plasma membrane receptor stimulation, extracellular Ca²⁺, and/or ROS/RNS elevation and amplify them through cross-talk between themselves (29, 51). Next, these signal transduction pathways transmit into mitochondrial matrix by changing the function

of mitochondrial ion channels/transporters, especially by changing the activities of Ca²⁺-influx mechanisms at the IMM (Figs. 1 and 2). Ca²⁺ accumulation into mitochondrial matrix through Ca²⁺ channels/transporters at the IMM affects the efficiency of electron flow, ATP production, and O₂⁻ generation at the ETC either directly (*e.g.*, by changing complex V activity) (174) or indirectly (through changing tricarboxylic acid [TCA] cycle activity and $\Delta\Psi_m$) (63) (Fig. 2). Thus, mitochondrial ion channels/transporters that are responsible for Ca²⁺ influx and H⁺ pumps (ETC) at the IMM are important regulators for mitochondrial redox signaling (Figs. 1 and 2). In ETC and Mitochondrial ROS Generation section, we describe the interaction between mitochondrial ROS generation and ETC function. We show the detailed mechanism of how the ETC regulates mitochondrial ROS generation and also of how ROS itself affects ETC function. Next, we summarize updated information of how the mitochondrial Ca²⁺ influx mechanism

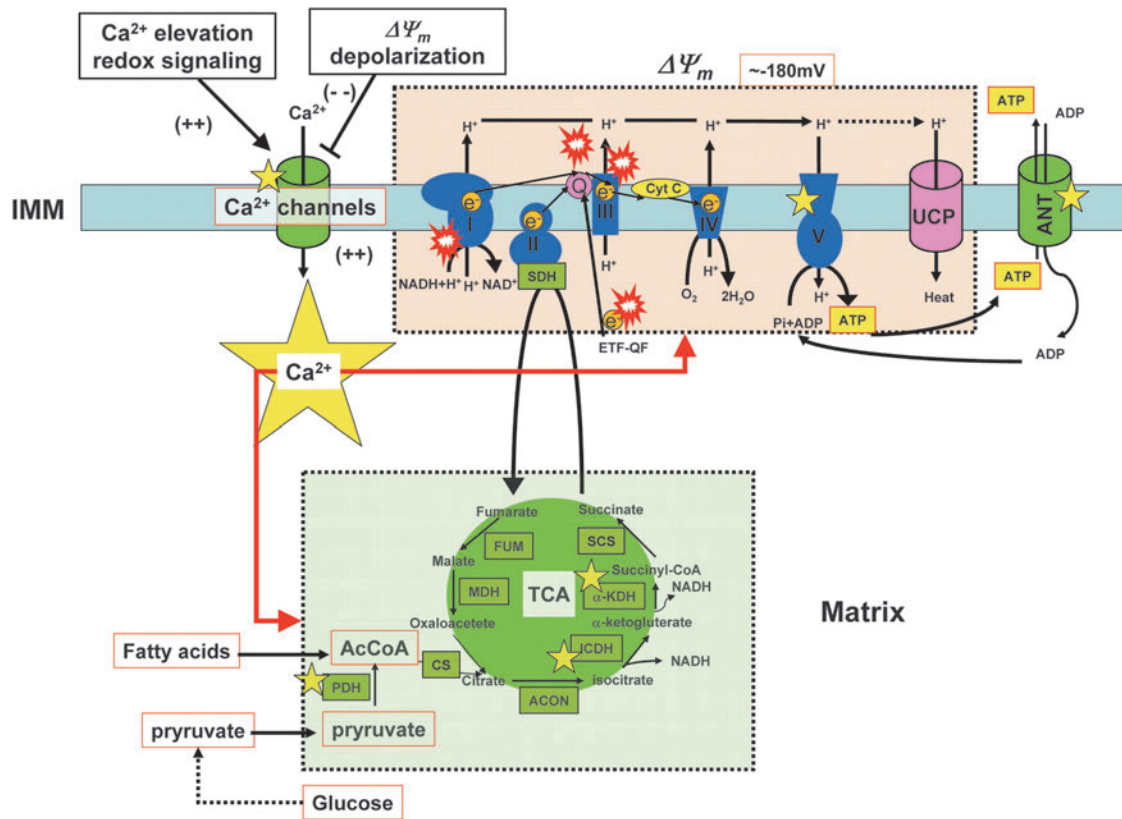


FIG. 2. Mitochondrial ETC and mitochondrial ROS generation. In a healthy eukaryotic cell, mitochondria generate more than 90% of the total intracellular ATP through the TCA cycle (green area) and OXPHOS (pink area). During OXPHOS, O_2^- is continuously produced as a primary oxygen free radical in mitochondria. NADH is produced from cytosolic glucose oxidation and the TCA cycle and passes electrons to NADH dehydrogenase (complex I). Then, complex I transfers electrons to CoQ10. CoQ10 can also receive electrons from succinate (complex II) and glycerol-3-phosphate dehydrogenase (NAD⁺). Electrons from reduced CoQ10 are then transferred to cytochrome oxidoreductase (complex III). Next, complex III transfers the electrons to cytochrome c and the electron transport continues COX (complex IV) and molecular oxygen. Electron transfer processes through complexes I, III, and IV produce Δp , which, in turn, are used to drive ATP synthase (complex V). When Δp increases, electron transport in complex III is partially inhibited and results in an increased backup of electrons to CoQ10 for binding to molecular oxygen, leading to the generation of O_2^- . In addition, when the electron-transport efficiency at complex I is suddenly inhibited or complex I activity is changed, this mechanism also generates O_2^- . Red “explosion” symbols indicate places where O_2^- production occurs. Yellow star symbols indicate enzymes/channels that are regulated by $[Ca^{2+}]_{mt}$. ETF-QO, electron transferring flavoprotein-quinone oxidoreductase; UCP, uncoupling protein; ANT, adenine nucleotide translocase; PDH, pyruvate dehydrogenase; CS, citrate synthase; ACON, aconitase; ICDH, isocitrate dehydrogenase; α -KDH, α -ketoglutarate dehydrogenase; SCS, succinyl-CoA synthetase; SDH, succinate dehydrogenase; FUM, fumarate; MDH, malate dehydrogenase; TCA, tricarboxylic acid; OXPHOS, oxidative phosphorylation; $[Ca^{2+}]_{mt}$, mitochondrial matrix Ca^{2+} concentration; CoQ10, coenzyme Q10; COX, cytochrome c oxidase; cytC, cytochrome C. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

regulates mitochondrial ROS generation (see Mitochondrial Ca^{2+} Influx Mechanism and Mitochondrial ROS Generation section). Here, we also discuss the possibility of whether ROS signaling itself can modulate mitochondrial Ca^{2+} channels/transporters through redox-dependent PTMs.

As briefly mentioned in the Introduction section, not only ROS but also RNS exerts a broad spectrum of functions in mitochondria (28, 29, 33, 58, 195). Compared to O_2^- , NO has more low reactivity and hydrophobicity, which enables NO to diffuse large distances both within and between cells (176). Therefore, the importance of spatiotemporal control of NO production is highlighted by the localization of NOS isoforms and their subcellular localization-dependent activities (58, 118, 126). Three types of NOSs (neuronal NOS [nNOS], inducible NOS [iNOS], and endothelial NOS [eNOS]) are well characterized, associated with various biological membranes (e.g., plasma membrane, nucleus, Golgi, and sarco/endo-

plasmic reticulum [SR/ER]), and activated through different mechanisms (58, 118, 126). It is reasonable to assume that NO synthesized at the cytosol can diffuse into mitochondria and modulate their functions. Indeed, cytochrome c oxidase (COX) (complex IV) is one of the main targets of NO, and the inhibition of complex IV by NO binding has been well established (28, 194). In the 1990s, several laboratories proposed that mitochondria also serve as one of the cellular sources for NO production through mtNOS (Figs. 1 and 3), but the existence of mtNOS is still controversial (see also Introduction section). In Regulation of ETC Activity by Mitochondrial NO Signaling section, we will describe the detailed mechanism of how NO regulates mitochondrial ROS generation by changing ETC function and also discuss the controversy of the existence of mtNOS. In Interaction of NO, Mitochondrial Ca^{2+} , and ROS Generation section, we summarize the cross-talk between Ca^{2+} , NO, and ROS in mitochondria.

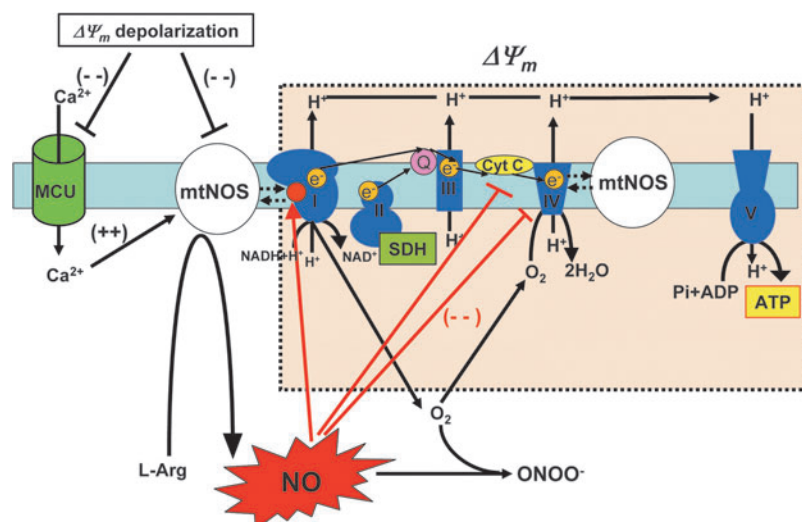


FIG. 3. Regulation of ETC activity by mitochondrial NO signaling. The existence of mtNOS and the production of NO in matrix are still controversial (see Introduction, Overview: Mitochondrial Ion Channels/Transporters, ETC, and Mitochondrial Redox Signaling, Regulation of ETC activity by mitochondrial NO signaling, and Interaction of NO, mitochondrial Ca^{2+} , and ROS generation sections). NO diffused from cytosol to matrix or produced in mitochondrial matrix by mtNOS can reversibly interact with (complex IV) in competition with oxygen. NO inhibits the electron flow between cytochrome b and cyt c at complex III. NADH dehydrogenase (complex I) receives S-nitrosylation (red circle), which shows reversible inhibition of complex I activity. Therefore, mitochondrial NO interferes with ETC activity (electron flow), and excessive mitochondrial NO production results in a decrease of ATP production, depolarization of $\Delta\Psi_m$ and an increase in ROS generation. There are several reports showing that this tight functional coupling of NO and complex I or IV is derived from the physical interaction between mtNOS and complex I or IV. Complex I, NADH dehydrogenase; complex II, SDH; complex III, cytochrome bc_1 complex; complex IV, COX; complex V, ATP synthase; $\Delta\Psi_m$, mitochondrial membrane potential. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

At the IMM, in addition to Ca^{2+} channels/transporters, several K^+ channels, including $\text{mitoK}_{\text{ATP}}$ and mitochondrial Ca^{2+} -activated K^+ channel (mitoK_{Ca}), are present. These channels indirectly modulate ROS generation in pathological conditions such as ischemic/reperfusion (I/R) in the heart (36, 124, 127, 128). Opening of $\text{mitoK}_{\text{ATP}}$ and mitoK_{Ca} , either by cellular signal transduction or by extracellular stress such as I/R, depolarizes $\Delta\Psi_m$, which reduces the driving force for Ca^{2+} influx, thereby attenuating mitochondrial Ca^{2+} overload and excessive ROS generation. Consequently, prevention of matrix Ca^{2+} overload and ROS over-generation protects the heart against cell death (cardioprotection). In ETC and Mitochondrial Redox Signaling section, we summarize updated information regarding the molecular identities of these K^+ channels and discuss the possibility of redox-dependent modulation of mitochondrial K^+ channels.

Under pathological conditions, the release of ROS/RNS and proapoptotic proteins from mitochondria act as important amplifiers for accelerating the ROS-induced mitochondrial ROS generation as well as cell death signaling. These redox release channels include mPTP (see Mitochondrial Permeability Transition Pore and Redox Signaling section) and IMAC (see IMAC and Redox Signaling section). Voltage-dependent anion channels (VDAC) are redox-sensitive channels that are located at the OMM and regulate OMM permeability (see VDAC and Redox Signaling section) (64). O_2^- can trigger VDAC-dependent OMM permeabilization, which subsequently activates apoptotic cascades (99). These channels receive redox-dependent PTMs and often act as an amplifier or positive feedback core to accelerate the patho-

logical processes due to their dual role as sensors/regulators of redox signaling.

Overview: Mitochondrial Ion Channels/Transporters and Redox-Dependent PTMs

ROS/RNS can alter protein structures/functions, including channel-subunit proteins, by modifying critical amino-acid residues, including (i) cysteine (30, 144) and methionine oxidation (30, 74, 157), (ii) cysteine S-glutathionylation (72, 117), (iii) cysteine disulfide bonds (175), (iv) cysteine surhydration (136), (v) cysteine S-nitrosylation (70, 71, 104), (vi) tyrosine 3-nitration (102), and (vii) dityrosine formation (175) (Fig. 4). In addition, these redox-dependent PTMs are known to change the biophysical properties of ion channels/transporters that are located at plasma membranes or organelle membranes (41, 65, 74, 115) such as their activation/deactivation profiles, Ca^{2+} or voltage dependency, and interaction affinities between regulatory proteins (Fig. 5). In addition, ion channels/transporters are frequently regulated through phosphorylation by upstream protein kinases (45), whose activities are modulated by redox-dependent PTMs (Fig. 1). For instance, protein kinase A (PKA), protein kinase C (PKC) (60) and protein kinase D (PKD) (169), and Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) (50) are activated either by directly receiving redox-dependent PTMs (for PKA, PKC, and CaMKII) or by the redox-sensitive upstream kinases (for PKC, PKD, and CaMKII), which can ultimately modulate voltage-gated Ca^{2+} channels at the plasma membrane (186). Therefore, it is conceivable that the mitochondrial ion channels/transporters are also subject to redox-dependent PTMs

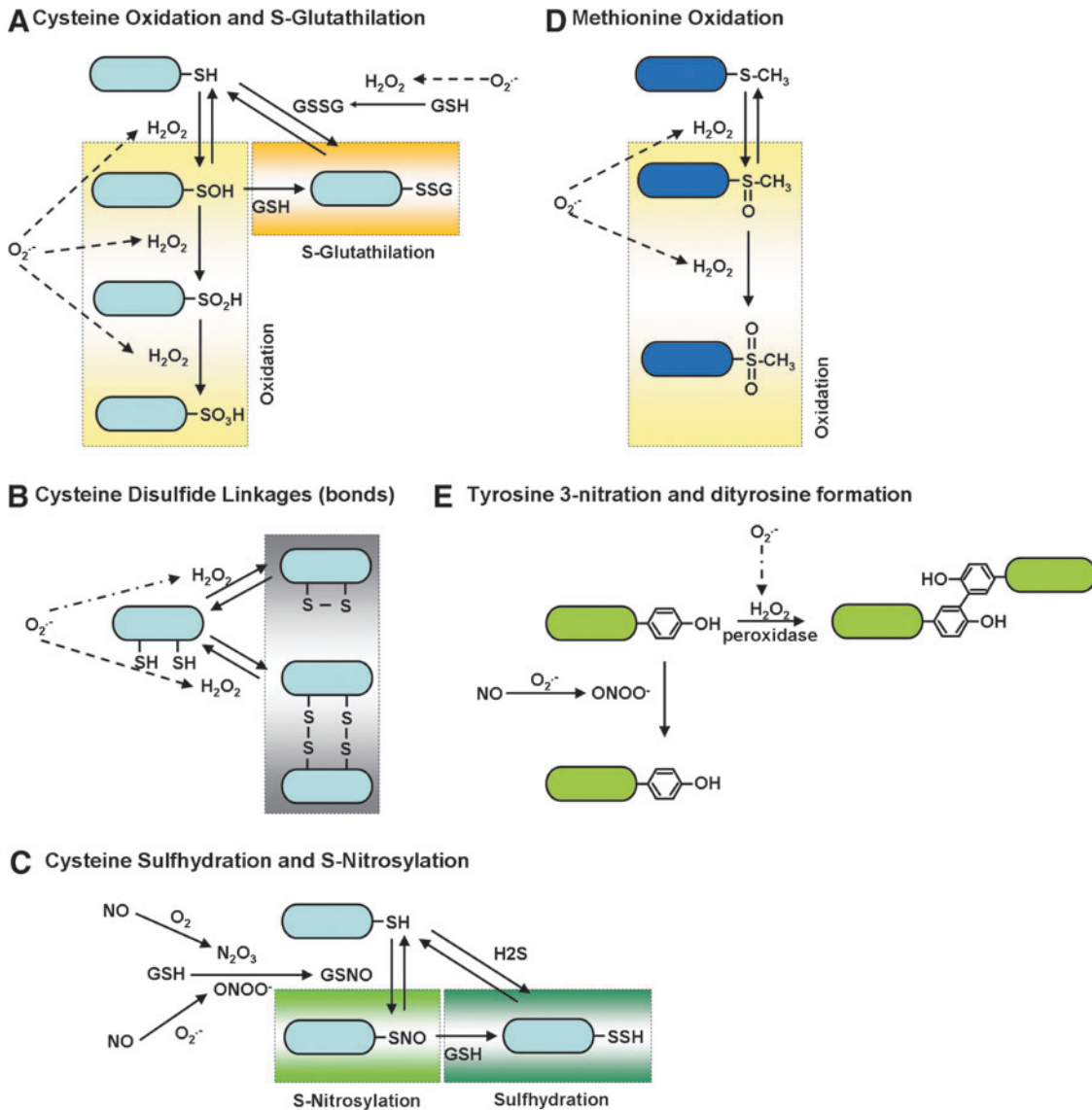


FIG. 4. Redox-dependent PTMs. The redox-dependent PTMs are shown. These include cysteine and methionine oxidation (A, D), cysteine *S*-glutathionylation (A), cysteine disulfide bonds (B), cysteine surhydration (C), cysteine *S*-nitrosylation (C), tyrosine 3-nitration (E), and dityrosine formation (E). GSH, glutathione; GSSG, glutathione disulfide; GSNO, *S*-nitrosoglutathione; H₂S, hydrogen sulfide. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

and phosphorylation through redox-sensitive kinases, which eventually regulate mitochondrial ROS/RNS homeostasis (see Overview: Mitochondrial Ion Channels/Transporters, ETC, and Mitochondrial Redox Signaling section and Fig. 1). Since the molecular identities of the main mitochondrial ion channels/transporters have just been discovered (see Introduction section), investigation of the PTMs of these proteins has just started and the role of mitochondrial redox regulation has begun to emerge (see Mitochondrial Ca²⁺ Influx Mechanism and Mitochondrial Redox Signaling, Mitochondrial K⁺ Channels and Mitochondrial Redox Signaling, Mitochondrial Permeability Transition Pore and Redox Signaling, IMAC and Redox Signaling, and VDAC and Redox Signaling sections). In addition, the components of the ETC are also targets of redox-dependent PTMs, which influence the efficiency of electron flow, ATP synthesis and ROS production (see ETC and Mitochondrial Redox Signaling section).

ETC and Mitochondrial Redox Signaling

ETC and mitochondrial ROS generation

ROS are generated by several different cellular sources, which include membrane-associated NADPH oxidase, cytosolic xanthine and xanthine oxidase, and the ETC at the IMM (177) (see Introduction section). Among those, mitochondria are the main source of ROS and play a central role in redox signaling in all cell types/tissues (Figs. 1 and 2). O₂⁻ is the primary oxygen free radical *via* the slippage of an electron from the ETC to molecular oxygen during OXPHOS (177) (see Overview: Mitochondrial Ion Channels/Transporters, ETC, and Mitochondrial Redox Signaling section and Fig. 2).

The most prominent contribution of mitochondria to cellular metabolism is their capacity to generate ATP through the TCA cycle and OXPHOS (Fig. 2). The main driving force of OXPHOS is known as "chemiosmosis" (63). Chemiosmosis is

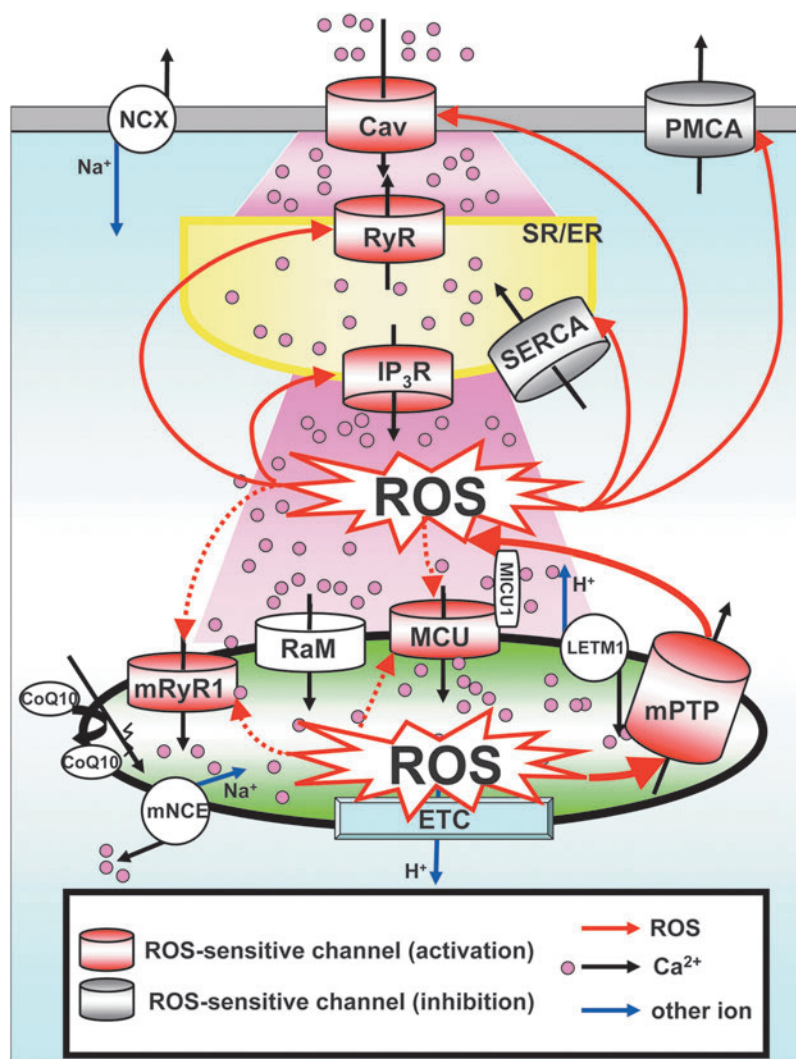


FIG. 5. ROS-dependent regulation of cellular Ca^{2+} handling and ROS-induced ROS generation. Schematic diagram of ROS-dependent regulation of cellular Ca^{2+} handling and ROS-induced ROS generation. OMM structure is abbreviated in this figure. Red channels/transporters can be activated by the redox-dependent PTM. Gray structure is abbreviated in this figure. Red channels/transporters can be activated by the redox-dependent PTM. Gray channels/transporters can be inhibited by the redox-dependent PTMs. The redox modulations of white channels/transporters are unknown (no report) or still controversial. Voltage-gated Ca^{2+} channels (Cav) at the plasma membrane are phosphorylated by redox-dependent kinases and activated. RyR and IP_3R at SR/ER are activated by redox-dependent PTMs, which increase Ca^{2+} release from SR/ER. SERCA is inhibited by irreversible oxidative modifications. PMCA is inhibited by either direct or indirect redox-dependent modifications. Thus, redox signaling generally increases cytosolic $[\text{Ca}^{2+}]_c$. This $[\text{Ca}^{2+}]_c$ elevation feeds back to mitochondria through an increase in the Ca^{2+} influx to mitochondrial matrix, which results in a positive feedback loop of ROS-induced ROS generation. mRyR1 and MCU, which are responsible for mitochondrial Ca^{2+} influx mechanism, are capable of receiving redox-dependent modulation. Excessive ROS/RNS are released through mPTP or IMAC (not shown in this figure) to the cytosol. Mitochondrial Ca^{2+} efflux is mainly regulated by an mNCE. During chronic heart failure, elevation of cytosolic $[\text{Na}^+]_c$ accelerates mitochondrial Ca^{2+} efflux by mNCE and blunted $[\text{Ca}^{2+}]_{mt}$ accumulation, followed by an increase in the mitochondrial ROS level through the reduction of the NADPH-dependent antioxidative capacity at the matrix to control the mitochondrial H_2O_2 level. Thus, elevated $[\text{Na}^+]_c$ and mNCE activity also contributes to the regulation of $[\text{Ca}^{2+}]_{mt}$ homeostasis and ROS production, especially during chronic heart failure (see detailed in Mitochondrial Ca^{2+} Influx Mechanism and Mitochondrial ROS Generation section). PMCA, plasma membrane Ca^{2+} ATPase; RaM, rapid mode of uptake; LETM1, leucine zipper-EF-hand containing transmembrane protein 1; NCX, $\text{Na}^+/\text{Ca}^{2+}$ exchanger at plasma membrane; SR/ER, sarco/endoplasmic reticulum; SERCA, SR/ER Ca^{2+} -ATPase; $[\text{Ca}^{2+}]_c$, cytosolic Ca^{2+} concentration; $[\text{Na}^+]_c$, Na^+ concentration; H_2O_2 , hydrogen peroxide; MCU, mitochondrial Ca^{2+} uniporter pore; NADPH, nicotinamide adenine dinucleotide phosphate; RyR, ryanodine receptor; mRyR1, mitochondrial ryanodine receptor type 1; IP_3R , IP_3 receptors. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

the movement of ions across a selectively permeable membrane, down their electrochemical gradient (protonmotive force: Δp), which is determined by both $\Delta\Psi_m$, and ΔpH components across the IMM ($\Delta p = \Delta\Psi_m + \Delta pH$) (92). This large driving force for H^+ influx (Δp) is used by the mitochondrial ATP synthase (complex V) to produce ATP (Fig. 2). OXPHOS through the ETC, which is a concerted series of redox reactions, is catalyzed by four multi-subunit enzymes in the IMM (complex I–IV) and two soluble factors, cytochrome c (cytC) and coenzyme Q10 (CoQ10), which function as electron shuttles within the IMS (Fig. 2). In a healthy eukaryotic cell, more than 90% of the total intracellular ATP is generated by mitochondria. The basic assumption of this chemiosmotic theory is derived from the important observation that the IMM is generally impermeable to ions, but it maintains the permeability of H^+ by its unique lipid composition of the IMM (39), which also contributes to maintaining the structure of cristae and enhances the efficiency of ETC activity, possibly through the facilitation of forming super complexes of the respiratory chain at the IMM (166). In the ETC, electrons transfer from electron donors such as NADH and CoQ10 to an electron acceptor O_2 through a series of redox reactions (Fig. 2). This reaction generates a H^+ gradient across IMM and complex I, III, and IV works as H^+ pumps. O'Rourke's group showed in cardiomyocytes that the majority of mitochondrial ROS production is from complex III (129), whereas Wei and Dirksen proposed that O_2^- from complex I triggers transient mPTP opening in cardiac and skeletal muscles (189). As mentioned in Introduction section, it has also been shown that mitochondrial ROS serves as an important signaling platform for T-cell receptor signaling activation and the regulation of T-cell homeostasis [see reviews (67, 120)]. Kaminski and Gulow's group demonstrated a crucial role of complex-I-mediated mitochondrial ROS production for T-cell receptor-mediated PKC θ activation (83, 84). They showed that the ETC switches from an ATP-producing to an oxidative signaling function on T-cell receptor activation, and this mechanism is possibly due to reverse electron transfer by a highly reduced pool of ubiquinone, which is a major mechanism of mitochondrial ROS generation *via* complex I (85). Meanwhile, Chandel's group demonstrated that ROS production from complex III is specifically required for increasing interleukin-2 expression during T-cell receptor activation (159). Thus, it still remains to be determined which sites (complex I or III) of ROS production dominantly regulate tissue-specific cellular redox signaling and function under physiological or pathophysiological conditions.

As mentioned earlier, the main route for the H^+ to flow into mitochondrial matrix, driven by the electrochemical gradient, is through complex V. Another route for the proton inflow is named "proton leak," attributed by uncoupling proteins, and this process can also modulate the ATP/ADP ratio (163) (Fig. 2). Proton leak is known to significantly contribute to the control of respiration in mitochondria in state 4 and to some extent in state 3. Therefore, uncoupling proteins also play an important role in sustaining proton leak, preventing excessive H^+ gradient, and, subsequently, avoiding excessive ROS production (88, 109).

By using mitochondrial-targeted circularly permuted yellow fluorescent protein, recent studies from our collaborating groups showed a "stochastic" and "transient" O_2^- burst from

either single or restricted clusters of interconnected mitochondria or isolated mitochondria, termed as a "mitochondrial superoxide flash" (mSOF) (188). Interestingly, this mSOF is usually associated with a transient depolarization of $\Delta\Psi_m$ (160, 189). mSOF generation is currently proposed as a result of a small increase in constitutive ROS production in mitochondria, which transiently opens a large channel mPTP to evoke transient $\Delta\Psi_m$ depolarization and subsequently, stimulates the ETC to produce a burst in O_2^- production. This idea is supported by the previous observations that the mPTP opens and closes transiently ("flickers") at its low conductance state (18, 66). Since observed mSOF frequencies vary widely across different cell types and the experimental conditions, our future studies will attempt to use these flashes as a biomarker for the cellular metabolic activity and the oxidative status in physiological and pathophysiological conditions (160). Further studies will also clarify the contribution of altered mSOF activity to ROS overproduction and metabolic dysfunction in a wide range of mitochondrial diseases and oxidative stress-related disorders. It should be also mentioned that the mSOF activity contains a small signal component from mitochondrial pH fluctuation, but much smaller than that from O_2^- (190). Future studies will also be directed toward inventing a novel O_2^- -sensitive biomarker to reduce its pH sensitivity.

Regulation of ETC activity by mitochondrial NO signaling

In conjunction with the hydrophobic nature of mitochondria as a cellular compartment, it is a reasonable idea that mitochondria are a significant cellular sink for NO (161) (see Overview: Mitochondrial Ion Channels/Transporters, ETC, and Mitochondrial Redox Signaling section). Indeed, since the discoveries of the biological function of NO (see Overview: Mitochondrial Ion Channels/Transporters, ETC, and Mitochondrial Redox Signaling section), cellular targets of NO have been extensively investigated and reversible binding of NO to COX (complex IV) in mitochondria has been well characterized (28, 194). Through investigation of NO function in mitochondria, it was proposed in the 1990s from several laboratories that mitochondria possess their own isoform of NOS, termed "mtNOS," which can produce NO in mitochondrial matrix similar to other NOS isoforms observed in the cytosol (Figs. 1 and 3) (13, 14, 56, 59, 91, 143, 172) [see reviews (28, 58, 194)]. However, a number of studies and comments show the conflicting evidence about the existence of mtNOS (28, 43, 96, 97, 173, 184, 185) (see Figs. 1 and 3). We include both types of papers in this review, as they provide important insights into the development of the knowledge of mitochondrial NO functions.

$[NO]_{mt}$ is around nanomolar ranges (3, 4, 58, 179, 194) and at this concentration of NO, it is able to compete with oxygen and interact with COX reversibly (3, 4, 58, 194), interfere with electron flow that results in a decrease in ATP production (62), depolarization of $\Delta\Psi_m$ (180, 181), and an increase in ROS generation (142, 156) (Fig. 3). Several studies showed that complex I is also a target for NO and receives S-nitrosylation (Fig. 4C), which causes reversible inhibition of complex I activity (25, 42, 55) (Fig. 3). Recently, Murphy's group demonstrated that the reversible inhibition of complex I by selective S-nitrosylation of Cys39 on the ND3 subunit during the

crucial first minutes of the reperfusion of ischemic hearts reduces oxidative damage and tissue necrosis by decreasing ROS production from mitochondria (40). Several groups reported that this tight functional coupling of NO and complex I or IV is derived from the physical interaction between mtNOS and complex I or IV (55, 140) (Fig. 3). Moreover, Poderoso's group reported that NO exerts inhibition of the electron flow between cytochrome b and cytC at complex III in addition to complex IV (142, 143). These findings indicate that NO can regulate ETC activity through direct binding of NO and/or the redox-dependent PTMs, which eventually change the efficiency of mitochondrial ROS generation.

Though the significant role of NO on ETC has been well recognized, there are a number of contradictory reports with regard to the existence and molecular identity of an mtNOS as shown earlier [see reviews (28, 58, 126, 194, 195)]. The diversity of the results is possibly derived from the technical issues used in the earlier studies, including (i) the use of isolated mitochondria and their purity; (ii) the reliability of biochemical and immunohistochemical assays using specific antibodies; and (iii) lack of suitable methods for measurement of mitochondrial NO apart from cytosolic NO. Recently, genetic approaches were also applied to clarify the molecular identity of a putative mtNOS. Through biochemical analysis, eNOS and iNOS are found in mitochondria (79), but the eNOS or iNOS knockout mice still possess NOS activity in mitochondria (86). Meanwhile, no signal was detected in mitochondria from nNOS-knockout mice, suggesting that nNOS is involved in mitochondrial NOS activity (86). This observation is supported by the recent report from the Ritter's group using nNOS-overexpression mice showing that nNOS overexpression causes nNOS translocation into mitochondria, which results in cardioprotection after I/R injury by reducing mitochondrial ROS generation (32). Putzke *et al.* proposed using nNOS α knockout mice that a splice variant of nNOS is mtNOS (146). However, further studies will be required to clarify whether there is functional nNOS activity in mitochondria in physiological conditions.

Mitochondrial Ca²⁺ Influx Mechanism and Mitochondrial Redox Signaling

Mitochondrial Ca²⁺ influx mechanism and mitochondrial ROS generation

As first shown in early studies between the 1960s and 1970s, Ca²⁺ uptake into the mitochondrial matrix stimulates ATP synthesis (66, 132). Ca²⁺-dependent ATP generation is explained as follows: (i) Three rate-controlling dehydrogenases of the TCA cycle are activated by Ca²⁺; (ii) Ca²⁺-dependent acceleration of TCA cycle increases the production of the primary electron donor for ETC (NADH); and (iii) complex V is activated by Ca²⁺ (Fig. 2) (63). In addition, regeneration of NADPH-dependent antioxidative capacity is also stimulated by Ca²⁺, because regeneration of NADPH requires products of the TCA cycle (193). At the resting state, the electrochemical driving force for Ca²⁺ uptake is provided by $\Delta\Psi_m$ across the IMM (see Fig. 2). The mitochondrial Ca²⁺ influx mechanism was initially considered a result of a single channel/transporter function (MCU) that was proposed more than 30 years ago, principally due to nearly complete inhibition by ruthenium red and lanthanides [see review (66)]. However, updated reports clearly show that additional Ca²⁺

channels/transporters, whose characteristics are different from the original MCU theory, also exist and, indeed, participate in the mitochondrial Ca²⁺ influx mechanism, including a rapid mode of uptake (RaM) (31, 168), Leucine zipper-EF-hand containing transmembrane protein 1 (LETM1) (81), CoQ10 (22), and mitochondrial ryanodine receptor (RyR) type 1 (mRyR1) (19, 20, 154) [see also review (132)] (Fig. 5).

If Ca²⁺ is taken into the mitochondrial matrix down its electrochemical gradient without transport of another ion such as through MCU (37, 90), there is a net transfer of two positive charges into matrix, resulting in a drop of $\Delta\Psi_m$ for each Ca²⁺ influx through MCU. However, Ca²⁺ accumulation by MCU is counteracted predominantly by mNCE (87, 128, 129, 149) to maintain mitochondrial matrix Ca²⁺ concentration ([Ca²⁺]_{mt}). Moreover, the Ca²⁺-stimulated respiration will not only compensate the loss of $\Delta\Psi_m$ by the efflux of H⁺ through the ETC but also produce a net gain of ATP. Thus, the physiological range of Ca²⁺ uptake into the mitochondrial matrix stimulates ATP synthesis without loss of $\Delta\Psi_m$. However, if sustained cytosolic Ca²⁺ concentration ([Ca²⁺]_c) elevation occurs, the [Ca²⁺]_c triggers excessive mitochondrial Ca²⁺ uptake (*i.e.*, mitochondrial Ca²⁺ overload), followed by $\Delta\Psi_m$ depolarization, a decrease in ATP production, acceleration of ROS generation, and activation of cell death pathways under various pathological conditions; such as under I/R of the heart (29, 101), excitotoxicity of neurons (131), vascular hypoxia (111), and inflammation in endothelial cells (153). Indeed, application of Ca²⁺ to isolated mitochondria stimulates ROS formation at the level of either complex I (167) or complex III (34) in the ETC, suggesting that the mitochondrial Ca²⁺-influx mechanism alone can activate mitochondrial ROS generation (Fig. 2). We also reported that blocking mitochondrial Ca²⁺ uptake by Ru360 can significantly inhibit mitochondrial ROS generation in intact striatal neurons under intense *N*-methyl-D-aspartate (NMDA) receptor activation (excitotoxicity), indicating that mitochondrial ROS increase requires Ca²⁺ entry into mitochondria (49). However, it is still not clear (i) which mitochondrial Ca²⁺ channel(s) is (are) responsible for the Ca²⁺ influx mechanism and (ii) how the Ca²⁺ that enters into mitochondria enhances ROS generation [see also reviews (29, 35)]. For the first question, Cheng's group demonstrated that the knocking down of MCU or its regulatory protein MICU1 markedly diminishes mSOF activity under the cytosolic Ca²⁺ elevation by hyperosmotic stress (75), suggesting that at least Ca²⁺ influx through MCU results in O₂⁻ generation in mitochondria. Moreover, Rizzuto's group showed that MCU-overexpressing cells were more efficiently killed after treatment with H₂O₂, indicating that mitochondrial Ca²⁺ influx through MCU enhances the effect of oxidative stress to mitochondria (46). Therefore, these observations support the initial idea that at least, mitochondrial Ca²⁺ influx through MCU regulates mitochondrial ROS generation, especially in pathophysiological conditions (Ca²⁺ overload). Future studies need to address whether Ca²⁺ influx through other mitochondrial Ca²⁺ channels/transporters (132) can influence mitochondrial ROS generation. For the second question, it is possible that different levels of [Ca²⁺]_{mt} (physiological modest increases or pathological excessive increases) lead to different mechanisms for ROS generation (29). For instance, stimulation of the TCA cycle and OXPHOS by physiological

Ca^{2+} increases would simply enhance ROS output by making the metabolic rate faster and consume more O_2 , which results in more electron leakage from the ETC (Fig. 2). The other possibility is that mitochondrial Ca^{2+} increases the NO level (e.g., through mtNOS) (see Interaction of NO, Mitochondrial Ca^{2+} , and ROS Generation section), which inhibits ETC efficiency as described in Regulation of ETC Activity by Mitochondrial NO Signaling section (Fig. 3). Finally, $[\text{Ca}^{2+}]_{\text{mt}}$ overload can lead to mPTP-mediated ROS generation.

In addition to the mechanism of mitochondrial ROS generation through excessive mitochondrial Ca^{2+} influx, a decrease in mitochondrial Ca^{2+} uptake can also increase mitochondrial ROS level in certain pathophysiological conditions. A few studies have attempted to show altered mitochondrial Ca^{2+} uptake during heart failure, but limited evidence suggests that mitochondrial Ca^{2+} uptake is reduced in failing cardiomyocytes (105, 116). O'Rourke and Maack extensively demonstrated that elevation of cytosolic Na^+ concentration ($[\text{Na}^+]_c$) in heart failure dictates mitochondrial ROS generation through a decrease in mitochondrial Ca^{2+} uptake (93, 105, 107) [see reviews (106, 122, 130)]. Since mitochondrial Ca^{2+} efflux is mainly regulated by an mNCE as described earlier, $[\text{Na}^+]_c$ accelerates mitochondrial Ca^{2+} efflux and blunted $[\text{Ca}^{2+}]_{\text{mt}}$ accumulation. They demonstrated using a guinea pig heart failure model (pressure overload model using aortic banding) that reduced $[\text{Ca}^{2+}]_{\text{mt}}$ decreases dehydrogenase activity and TCA cycle efficiency (see also Fig. 2), followed by sustained oxidation of NADH and NADPH (93, 105, 107). This, in turn, reduces the NADPH-dependent antioxidative capacity at the matrix to control the mitochondrial H_2O_2 level (8, 9) and increases the mitochondrial ROS level. They also discussed that a decrease in NADH and NADPH regeneration could also explain a mismatch in energy supply and demand during heart failure (106, 122). Thus, elevated $[\text{Na}^+]_c$ and mNCE activity also critically contributes to the regulation of $[\text{Ca}^{2+}]_{\text{mt}}$ homeostasis and ROS production especially during heart failure.

The next question is whether mitochondrial ROS itself can modify or modulate the mitochondrial ion channels/transporters that are responsible for Ca^{2+} influx through PTMs. Since the molecular identity of MCU channel pore (15, 46) and its regulatory proteins (110, 139, 141) have just been discovered, the information to answer this question is still quite limited. There is still no report that MCU or its regulatory proteins possess these PTMs through redox signaling and change its channel functions. Anderson's group recently reported that there are two CaMKII phosphorylation candidate motifs at the N-terminus of MCU. These nonphosphorylation mimic MCU mutants do not respond to CaMKII-dependent MCU current activation under mitoplast patch clamp, suggesting that MCU can undergo PTMs by CaMKII (phosphorylation) and play a role in the regulation of MCU function (82). Since the oxidation of methionine residue in CaMKII can activate this kinase (50) (see also Fig. 4D), this report indicates that there is a tight interaction between ROS level and MCU channel function (Fig. 5). They also demonstrated that mitochondrial CaMKII inhibition in the heart is protective against I/R injury, myocardial infarction, and neurohumoral injury, suggesting that MCU current inhibition is beneficial for reducing cell death by certain pathological myocardial oxidative stresses. Further observations will be required to conclude whether oxidized CaMKII can be a direct

modulator for MCU through its direct phosphorylation and can regulate mitochondrial ROS levels in heart failure.

The next possible candidate for a redox-sensitive Ca^{2+} influx (mitochondrial Ca^{2+} channel) at mitochondria is mRyR1 (19, 20, 154). mRyR1 was discovered as the first mitochondrial Ca^{2+} influx mechanism with a known molecular identity (19, 20, 154). It is well established that redox-dependent PTMs regulate the activity of RyRs localized at cardiac and skeletal SR, which shows enhanced Ca^{2+} leak from SR. RyRs (115) at SR received disulfide bond, S-nitrosylation, S-glutathionylation, cysteine oxidation (sulfonic acid), and phosphorylation by oxidized CaMKII (123) (see also Fig. 4). Therefore, it is possible that the redox-dependent PTMs of mRyR1 can contribute to the $[\text{Ca}^{2+}]_{\text{mt}}$ overload in pathophysiological conditions (Fig. 5). Further studies will be required to determine the PTMs of mRyR1 and to characterize their physiological and pathophysiological relevance to mitochondrial Ca^{2+} uptake.

Unlike mitochondrial channels, the redox modification of Ca^{2+} channels in the cytosolic Ca^{2+} cycling have been well established (Fig. 5) [see reviews (23, 73)]. For instance, voltage-gated Ca^{2+} channels located at the plasma membrane are phosphorylated by oxidized CaMKII, and Ca^{2+} influx from the extracellular space to the cytosol increases (164, 165). Ca^{2+} release channels at SR/ER, RyR, and inositol 1,4,5-trisphosphate receptors (IP_3R) are activated by a redox-dependent PTM (73). SR/ER Ca^{2+} -ATPase (SERCA) can be modified by cysteine oxidation or tyrosine nitrosylation (Fig. 4A, E) (155). Low levels of ROS reversibly increase SERCA activity through oxidation, but higher levels of ROS cause its inactivation as a result of irreversible oxidative modifications (Fig. 4A). Plasma membrane Ca^{2+} ATPase (PMCA) is inhibited through either its direct oxidation (Fig. 4A) or methionine oxidation in its binding partner calmodulin (Fig. 4D) (73). Thus, redox signaling generally increases $[\text{Ca}^{2+}]_c$ through (i) facilitation of Ca^{2+} influx through the plasma membrane, (ii) enhancing Ca^{2+} release from SR/ER, and (iii) inhibiting Ca^{2+} export from cytosol (Fig. 5). This $[\text{Ca}^{2+}]_c$ elevation feedbacks to mitochondria through an increase in the Ca^{2+} influx to mitochondrial matrix, which results in a positive feedback loop of ROS-induced-ROS generation. In addition, as mentioned earlier, a decrease in the mitochondrial Ca^{2+} influx (by the elevation of $[\text{Na}^+]_c$ and mNCE function) can also increase mitochondrial ROS level through the reduction of NADPH-dependent antioxidative capacity in the matrix.

Therefore, the cross-talk between mitochondrial ROS regulation and cellular Ca^{2+} cycling determines the amplitude of cellular redox signaling.

Interaction of NO, mitochondrial Ca^{2+} , and ROS generation

As described in Introduction, Overview: Mitochondrial Ion Channels/Transporters, ETC, Mitochondrial Redox Signaling, the existence and functional role of mtNOS is highly controversial. It is still challenging to reach an unequivocal conclusion using currently available approaches and determine whether mitochondria contain NOS, whether this NOS is able to produce physiologically relevant amounts of NO, and whether there are tissue-specific distribution and functions of mtNOS. In this section, we introduce recently published reports that attempt to characterize the interaction

between mitochondrial NO level, $[Ca^{2+}]_{mt}$, and ROS generation on the assumption that NOS activity exists in mitochondria.

Several groups reported that mtNOS has similar characteristics to that of cytoplasmic NOS isoforms. They proposed that the generation of NO by mtNOS is Ca^{2+} dependent and requires O_2 and L-arginine (47, 58, 194). Indeed, blocking the MCU by extra-mitochondrial $[Mg^{2+}]$ (196) or ruthenium red efficiently inhibits the mitochondrial NO level (47, 48), indicating that mitochondrial Ca^{2+} influx plays a role in maintaining the mitochondrial NO level. However, it is still in question whether this effect is mediated through mtNOS activity. In addition, depolarization of $\Delta\Psi_m$ by uncouplers strongly decreases the mitochondrial NO level (48, 86), suggesting a tight regulatory interaction between the mitochondrial NO level and $\Delta\Psi_m$ (180), but again, there is no direct observation that $\Delta\Psi_m$ can regulate mtNOS activity. Thus, these observations indicate that Ca^{2+} -induced NO elevation, in part, contributes to the Ca^{2+} influx-induced ROS generation through NO-dependent modification of ETC activity (see ETC and Mitochondrial Redox Signaling and Mitochondrial Ca^{2+} Influx Mechanism and Mitochondrial ROS Generation sections) (Figs. 2 and 3). However, it should be noted that most of the reports in this section measured mitochondrial NOS activity and/or NO level under cytosolic and mitochondrial Ca^{2+} overload, suggesting that the interaction of mitochondrial NO and Ca^{2+} plays some role in mitochondrial ROS production under pathophysiological conditions, such as I/R in the heart. Further studies are required to clarify the role of mitochondrial NO in mitochondrial Ca^{2+} homeostasis and ROS production under physiological conditions.

Mitochondrial K^+ Channels and Mitochondrial Redox Signaling

In cardiomyocytes, both $mitoK_{ATP}$ and $mitoK_{Ca}$ play key roles in cardioprotection, especially in ischemic preconditioning (IPC) (124, 128). The opening of $mitoK_{ATP}$ and $mitoK_{Ca}$ depolarizes $\Delta\Psi_m$, which reduces the driving force for Ca^{2+} influx, thereby attenuating the $[Ca^{2+}]_{mt}$ overload. Consequently, prevention of $[Ca^{2+}]_{mt}$ overload inhibits mitochondrial ROS generation (Figs. 1–3), mPTP opening (see next section: Mitochondrial Permeability Transition Pore and Redox Signaling) and protects against heart cell death. Several endogenous substances such as adenosine and bradykinin can reduce infarct size by activation of $mitoK_{ATP}$ channels in a PKC-dependent manner. Adrenomedullin, a potent vasodilator peptide, potentiates the opening of $mitoK_{Ca}$ by PKA activation. Treatment with adrenomedullin before ischemia results in the reduction of infarct size *via* a PKA-mediated activation of $mitoK_{Ca}$ channels. Thus, some endogenous substances can confer cardioprotection *via* PKA- or PKC-mediated activation of $mitoK_{ATP}$ or $mitoK_{Ca}$ channels (124, 128). As mentioned earlier, since PKA and PKC are redox-sensitive kinases, it is a reasonable idea that the activities of both $mitoK_{ATP}$ and $mitoK_{Ca}$ can be regulated by redox signaling through phosphorylation by PKA and/or PKC under IPC. Moreover, $mitoK_{ATP}$ can receive redox-dependent PTMs, indicating that the redox modulation of $mitoK_{ATP}$ is one of the key mechanisms for the cardioprotective effect in IPC (147). The simplest prediction for the molecular identity of $mitoK_{ATP}$ was that it is identical to K_{ATP} at the plasma membrane: a

protein complex with an inward rectifying K^+ channel (Kir6.2) as a pore region and a sulfonylurea receptor (SUR2A). Finally, O'Rourke's group has recently identified that one of the splice variants of the renal outer medullary K^+ channel (Kir1.1) is a suitable candidate for the pore-forming subunit of $mitoK_{ATP}$ using a mitochondrial proteomic approach (54). This novel finding will pave the way for a great opportunity to study the molecular basis of redox-sensitive PTMs in $mitoK_{ATP}$.

The large-conductance Ca^{2+} -activated K^+ channels (BK channels) are also biochemically detectable in the IMM of the brain and cardiomyocytes (124, 128). Therefore, the current proposed molecular identity of $mitoK_{Ca}$ is identical or similar to the BK channels. BK channels at the plasma membrane possess oxidative regulation (171). However, currently, there are no reports with regard to redox-sensitive PTMs in $mitoK_{Ca}$.

Mitochondrial Permeability Transition Pore and Redox Signaling

mPTP is one of the most widely studied mitochondrial channels/transporters as a critical effector/regulator of cellular redox signaling [see reviews (27, 68, 80, 103, 148)]. The properties of the mPTP are well defined, and mPTP activity is known to be redox, Ca^{2+} , $\Delta\Psi_m$, adenine nucleotide, inorganic phosphate, and pH sensitive (68, 95) (Fig. 6). For instance, under the conditions of $[Ca^{2+}]_{mt}$ overload especially when accompanied by the combination of high levels of ROS and $\Delta\Psi_m$ depolarization, sustained mPTP opening leads to the release of large amounts of Ca^{2+} and proapoptotic proteins from mitochondria, which subsequently leads to cell death (27, 80). The diameter of the pore is estimated to be large enough to pass molecules of approximately 1.5 kDa in their full open state. We and others reported cyclosporine A-sensitive large conductance channel activities (>1 nS single channel conductance) in the IMM (154). This channel also has transient and subconductance (<300 pS) states, and these channel properties contribute to an important physiological or pathological role by preventing $[Ca^{2+}]_{mt}$ overload working as a Ca^{2+} efflux mechanism (66) and/or by regulating mitochondrial ROS generation as described as an mSOF (188, 190). On mPTP opening, the IMM no longer maintains a barrier to protons, which leads to depolarization of $\Delta\Psi_m$, followed by the inhibition of ATP production. Due to its pore size, mPTP opening also results in equilibration of cofactors and ions across the IMM, including the release of accumulated Ca^{2+} . This will not only lead to the disruption of metabolic gradients between the mitochondria and the cytosol, but also influx of water concomitantly occurs and results in swelling of the mitochondria until the OMM eventually ruptures. The OMM rupture also facilitates the release of proapoptotic proteins and mitochondrial ROS, which potentially lead to apoptotic and necrotic cell death.

Despite numerous efforts, the exact molecular identity of mPTP is not conclusive (Fig. 6). Based on previous biochemical and pharmacological studies, the classical hypothetical model of mPTP is the supra molecular structure that is composed of (i) the VDAC in OMM; (ii) mitochondrial creatine kinase in IMS; and (iii) the adenine nucleotide translocase (ANT) in IMM and cyclophilin D (CypD) in the matrix. However, recent genetic studies using knockout mouse modes reveal that VDAC and ANT are not essential for pore

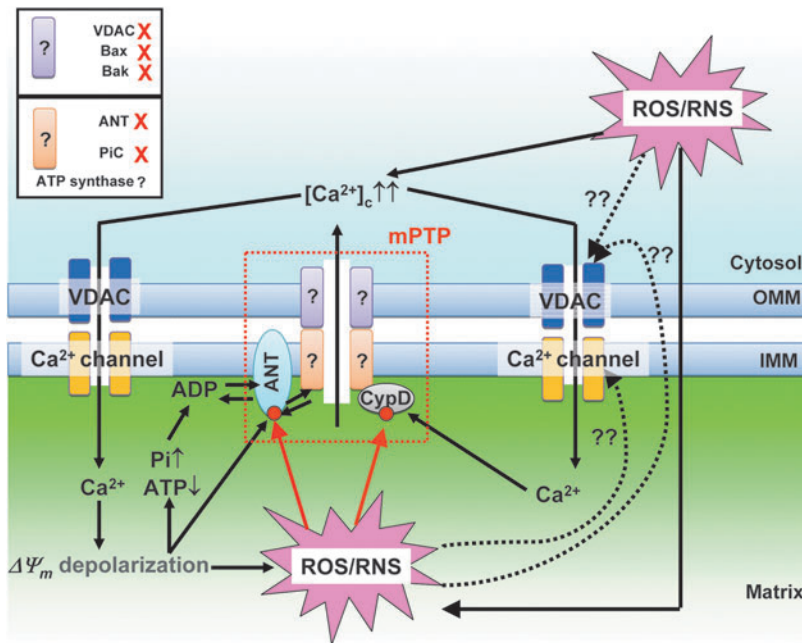


FIG. 6. Ca^{2+} - and redox-dependent regulation of mPTP. A hypothetical model of mPTP structure is shown at the middle (red dot line area). CypD and ANT are currently recognized as regulatory proteins of mPTP. CypD and ANT receive redox-dependent PTMs (red circles). CypD, cyclophilin D. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

opening and CypD potentially sensitizes the pore opening to Ca^{2+} (12). Therefore, these proteins have been currently established as regulatory rather than pore-forming components of the mPTP (94). Halestrap's group has recently proposed the mitochondrial phosphate carrier (PIC) as a pore component of the mPTP (68). However, PIC knockdown using siRNA in HeLa cells (65%–80% reduction of PIC) could not abolish mPTP opening, and an essential role for the PIC in mPTP formation still remains controversial (183). Finally, two recent publications have identified the mitochondrial ATP synthase as a possible molecular identity of the pore-forming structure of the mPTP (24, 61). Future studies will address the detailed molecular components of mPTP, including ATP synthase, and identify the critical site(s) for the direct redox-dependent PTMs of mPTP under physiological and pathophysiological conditions. Since recent publications showed that oxidation of α -subunits of ATP synthase at cysteine 294 can modulate its activity (187), this single cysteine will become a primary candidate site for investigating the PTM of mPTP.

There are several reports showing the redox-dependent PTM of mPTP regulatory proteins, CypD and ANT, but it is not well understood whether or how these modifications affect mPTP activity (for VDAC see VDAC and Redox Signaling section): Critical thiols on the matrix side of ANT, at Cys57, Cys160, and Cys257 regulate the bindings of ADP or CypD to ANT (114); the S-nitrosylation of Cys203 in CypD is necessary for the redox stress-induced activation of mPTP (121) (Fig. 6).

IMAC and Redox Signaling

IMAC is also an important sensor and regulator of ROS signaling [see review (129)]. Early studies of mitochondrial swelling showed that an anion-selective channel is present in IMM and later, it was named IMAC (57). IMAC is a benzodiazepine-sensitive channel and it shows ~ 100 pS single channel conductance (89). This channel is activated by matrix Mg^{2+} depletion or alkalization. We also reported that single channel recordings of the heart IMM show a variety of anion

selective conductance and the most prominent one is the centum picosiemen conductance channel, which matches the channel properties of IMAC (154). The single channel current-voltage curve of IMAC exhibits a linear relation, but the open probability of IMAC is higher in positive voltages, thereby showing an outward rectifying whole-mitoplast current (154). IMAC has slight anion selectivity and small molecules such as chloride, bicarbonate, phosphate, succinate, citrate, and ATP can pass through the IMAC pore (57).

The physiological role of IMAC is still unknown, because IMAC only appears to conduct ions under alkaline matrix conditions. However, this anion efflux through IMAC enables mitochondria to restore their normal volume after pathological swelling. The role of IMAC in pathophysiological conditions was well studied, especially in cardiomyocytes, by O'Rourke's group [see reviews (2, 5, 21, 192)]. They showed that photoactivation of single mitochondrion in cardiomyocytes using two-photon microscopy induces whole-cell wide oscillation of $\Delta\Psi_m$ and ROS generation (7). In this experimental model, they found that the O_2^- leaked through IMAC at the photoactivated mitochondrion and stimulated the opening of IMAC at the neighboring mitochondria. This series of reactions (regenerative ROS-induced IMAC opening in the neighboring mitochondria) through the mitochondrial network can reach a critical point that evokes whole-cell-wide oscillation of $\Delta\Psi_m$ and ROS generation. Moreover, this metabolic instability (or metabolic oscillation) induces an oscillatory activation of sarcolemmal K_{ATP} channel, which results in periodic shortening of the cardiac action potential (7). In an *ex vivo* whole heart model, this mechanism provides a "metabolic sink" in the I/R regions to induce abnormal propagation of cardiac action potential and cause arrhythmias (1). A benzodiazepine receptor antagonist, 4-cholorodiazepam abolishes metabolic oscillation, mitochondrial ROS generation, and cardiac arrhythmia induced by reoxygenation, indicating that redox-dependent modulation of IMAC plays critical roles in ischemia/reperfusion-induced arrhythmia.

The current major limitation in the research in IMAC functions is that the molecular identity of this channel has not yet been identified. Recently, the families of voltage-dependent Cl^- channels and/or Cl^- intracellular channel 4 (CLIC4) are proposed as candidates for IMAC (52). Further investigation will be required for confirming that the CLIC4 is, indeed, responsible for IMAC activity. Though the molecular identity of IMAC is still unknown, functional observation suggests that this channel contains thiol groups (16). Pretreatment of thiol reactive agents, *N*-ethylmaleimide, mersalyl, and *p*-chloromercuribenzenesulfonate increases the IC_{50} values of IMAC inhibitors (e.g., H^+ , Mg^{2+} , and cationic amphiphiles) (17). Matrix glutathione level seems to be critical for IMAC activity, because the inhibition of mitochondrial glutathione uptake, the NADPH-dependent glutathione reductase, or the NADH/NADPH transhydrogenase triggers IMAC opening (6). These observations support the idea that there are redox-dependent regulations of IMAC activity. Future studies will be required to understand the IMAC molecular identity as well as the precise patterns of the redox-dependent PTM of this channel.

VDAC and Redox Signaling

VDAC is the most abundant protein in the OMM, and it serves as the primary route of entry and exit for metabolites and ions across the OMM [see review (64)]. VDAC controls the diffusion of O_2^- from the IMS to the cytosol (69). Moreover, VDAC itself is a putative target of O_2^- , and O_2^- can modulate its function (108).

VDAC channels have a highly conserved homology with bacterial porins and can exist in a variety of functional states that differ in their ability to pass nonelectrolytes and conduct ions. VDAC gating has a voltage-dependent profile (162). VDAC shows ~ 3 nS in 1 M NaCl in the full conductance "open" state (152). In the open state, it shows a significant preference for anions and especially favors metabolic anions. The closed state favors cations. The permeability of VDAC to small cations by free diffusion includes K^+ , Na^+ , and Ca^{2+} (see also Fig. 6). Ca^{2+} ion permeates through both the open and closed states of VDAC, because the ion selectivity at the open state is rather poor (152). Nonelectrolytes (<6 kDa) can also pass through the open channel, enabling the passage of metabolites such as ATP, ADP, and inorganic phosphate (150).

As mentioned in Mitochondrial Permeability Transition Pore and Redox Signaling section, VDAC is not essential for the opening of mPTP, but its opening or closure is one of the important factors for the regulation of cell death signaling (Fig. 6). VDAC interacts with the apoptosis regulators, Bcl2-family members, including Bak, Bad, tBid, and Bcl-xL, which can change the VDAC channel activity (128, 152). For instance, Bax interaction can induce a novel high-conductance state of VDAC that permits cytC to escape from the IMS. Madesh and Hajnoczky found another mechanism for the VDAC-dependent permeabilization of the OMM by O_2^- . They showed in permeabilized hepatocytes that O_2^- treatment can elicit the proapoptotic protein cytC to be released from mitochondria through VDAC, whereas H_2O_2 cannot. This O_2^- -induced cytC release through VDAC is an independent phenomenon of Ca^{2+} -dependent mPTP opening. Moreover, O_2^- triggers apoptosis *via* VDAC-dependent OMM permeabilization without apparent contribution of

proapoptotic Bcl2-family proteins. However, the detailed mechanism of how O_2^- modulates VDAC function remains an open question. There are two possible mechanisms: (i) O_2^- directly adds PTMs to VDAC when released to the cytosol through this channel (69); and (ii) ROS, including O_2^- , targets VDAC *via* lipid oxidation by indirectly altering the lipid environment of VDAC (151). However, there is still no evidence to support these ideas.

Closing Remarks

Research conducted on the mitochondrial ion channels/transporters has been widely recognized as an important field in cell biology, even though their progress is relatively slow mostly due to the fact that their molecular identities remain unknown for a long period of time. Recent advances in the cloning of various mitochondrial ion channels/transporters have provided essential information for investigating the regulatory mechanisms underlying redox-dependent PTMs of the structure and function of these channels/transporters. This new knowledge is crucial for our understanding of the fundamental roles of mitochondrial ion channels/transporters in cell metabolism and signaling under healthy and diseased states. Furthermore, it will lead us to design or discover more specific inhibitors/activators to each channel/transporter that will have the potential to turn into therapeutic drugs for treating diseases caused by mitochondria-mediated oxidative stress. Meanwhile, we need to keep in mind that the molecular identities of the majority of mitochondrial ion channels/transporters are still unknown, which will limit our understanding of the whole picture of their roles as sensors and regulators of cellular redox signaling.

In conclusion, cardiac mitochondrial ion channels/transporters are crucial sensors and regulators for mitochondrial and cellular redox signaling. Revealing the molecular identities and biophysical and biochemical properties of mitochondrial ion channels/transporters will provide us with new strategies for antioxidation therapies targeting various human diseases.

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Abbreviations Used

α -KDH	= α -ketoglutarate dehydrogenase
$\Delta\psi_m$	= mitochondrial membrane potential
ACON	= aconitase
ANT	= adenine nucleotide translocase
BK channel	= large-conductance Ca ²⁺ -activated K ⁺ channel
[Ca ²⁺] _c	= cytosolic Ca ²⁺ concentration
[Ca ²⁺] _{mt}	= mitochondrial matrix Ca ²⁺ concentration
CaMKII	= Ca ²⁺ /calmodulin-dependent protein kinase II
CLIC4	= Cl ⁻ intracellular channel 4
CoQ10	= coenzyme Q10
COX	= cytochrome c oxidase
CS	= citrate synthase
CypD	= cyclophilin D
cytC	= cytochrome C
eNOS	= endothelial NOS
ER	= endoplasmic reticulum
ETC	= electron transport chain
ETF-QO	= electron transferring flavoprotein-quinone oxidoreductase
FUM	= fumarase
GSH	= glutathione
GSNO	= S-nitrosoglutathione
GSSG	= glutathione disulfide
H ⁺	= proton
H ₂ O ₂	= hydrogen peroxide
H ₂ S	= hydrogen sulfide
ICDH	= isocitrate dehydrogenase
IMAC	= inner membrane anion channel
IMM	= inner mitochondrial membrane
IMS	= mitochondrial intermembrane space
iNOS	= inducible NOS

Abbreviations Used (Cont.)

IP₃R = inositol 1,4,5-trisphosphate receptors
 IPC = ischemic preconditioning
 I/R = ischemic/reperfusion
 LETM1 = leucine zipper-EF-hand containing transmembrane protein 1
 MCU = mitochondrial Ca²⁺ uniporter pore
 MDH = malate dehydrogenase
 mitoK_{ATP} = mitochondrial ATP-sensitive K⁺ channel
 mitoK_{Ca} = mitochondrial Ca²⁺-activated K⁺ channel
 mNCE = mitochondrial Na⁺/Ca²⁺ exchanger
 mPTP = mitochondrial permeability transition pore
 mRyR1 = mitochondrial ryanodine receptor type 1
 mSOF = mitochondrial superoxide flash
 mtNOS = mitochondrial nitric oxide synthase
 [Na²⁺]_c = cytosolic Na²⁺ concentration
 NADPH = nicotinamide adenine dinucleotide phosphate
 NCX = Na⁺/Ca²⁺ exchanger at plasma membrane
 NMDA = *N*-methyl-D-aspartate
 nNOS = neuronal NOS
 NO = nitric oxide
 NOS = NO synthase

O₂⁻ = superoxide
 OMM = outer mitochondrial membrane
 ONOO⁻ = peroxynitrite
 OXPHOS = oxidative phosphorylation
 PDH = pyruvate dehydrogenase
 PIC = mitochondrial phosphate carrier
 PKA = protein kinase A
 PKC = protein kinase C
 PKD = protein kinase D
 PMCA = plasma membrane Ca²⁺ ATPase
 PTM = post-translational modification
 RaM = rapid mode of uptake
 RNS = reactive nitrogen species
 ROS = reactive oxygen species
 RyR = ryanodine receptor
 SCS = succinyl-CoA synthetase
 SDH = succinate dehydrogenase
 SERCA = SR/ER Ca²⁺-ATPase
 SR = sarcoplasmic reticulum
 TCA = tricarboxylic acid
 UCP = uncoupling protein
 VDAC = voltage-dependent anion channel