Am J Physiol Cell Physiol 287: C817–C833, 2004;
10.1152/ajpcell.00139.2004.

Invited Review

Calcium, ATP, and ROS: a mitochondrial love-hate triangle

Paul S. Brookes, Yisang Yoon, James L. Robotham, M. W. Anders, and Shey-Shing Sheu

Departments of Anesthesiology, Pharmacology, and Physiology and Mitochondrial Research Interest Group, University of Rochester Medical Center, Rochester New York 14642

Brookes, Paul S., Yisang Yoon, James L. Robotham, M. W. Anders, and Shey-Shing Sheu. Calcium, ATP, and ROS: a mitochondrial love-hate triangle. *Am J Physiol Cell Physiol* 287: C817–C833, 2004; 10.1152/ajpcell.00139.2004.—The mitochondrion is at the core of cellular energy metabolism, being the site of most ATP generation. Calcium is a key regulator of mitochondrial function and acts at several levels within the organelle to stimulate ATP synthesis. However, the dysregulation of mitochondrial Ca^{2+} homeostasis is now recognized to play a key role in several pathologies. For example, mitochondrial matrix Ca^{2+} overload can lead to enhanced generation of reactive oxygen species, triggering of the permeability transition pore, and cytochrome *c* release, leading to apoptosis. Despite progress regarding the independent roles of both Ca^{2+} and mitochondrial dysfunction in disease, the molecular mechanisms by which Ca^{2+} can elicit mitochondrial dysfunction remain elusive. This review highlights the delicate balance between the positive and negative effects of Ca^{2+} and the signaling events that perturb this balance. Overall, a "two-hit" hypothesis is developed, in which Ca^{2+} plus another pathological stimulus can bring about mitochondrial dysfunction.

mitochondria; reactive oxygen species; free radicals; apoptosis; neurodegeneration; ischemia; permeability transition

Ca^{2+} AND MITOCHONDRIAL PHYSIOLOGY

Mitochondrial oxidative phosphorylation (ox-phos) is the major ATP synthetic pathway in eukaryotes. In this process, electrons liberated from reducing substrates are delivered to O_2 via a chain of respiratory H^+ pumps. These pumps (complexes I–IV) establish a H^+ gradient across the inner mitochondrial membrane, and the electrochemical energy of this gradient is then used to drive ATP synthesis by complex V (ATP synthase) (136).

Chemically, the stepwise reduction of O_2 ($O_2 \rightarrow O_2^{-}\cdot \rightarrow H_2O_2 \rightarrow OH\cdot \rightarrow H_2O$) proceeds via several reactive oxygen species (ROS). These ROS can damage cellular components such as proteins, lipids, and DNA (70), but recent evidence also highlights a specific role in redox cell signaling for mitochondrial ROS (55, 203). In the fine balancing act of aerobic metabolism, mitochondrial ox-phos accomplishes the reduction of O_2 to H_2O while maximizing ATP synthesis and maintaining ROS production to only the amounts required for microdomain cell signaling (19, 87).

In addition to ATP synthesis, mitochondria are the site of other important metabolic reactions, including steroid hormone and porphyrin synthesis, the urea cycle, lipid metabolism, and interconversion of amino acids (39, 141). Mitochondria also play central roles in xenobiotic metabolism, glucose sensing/insulin regulation (113), and cellular Ca^{2+} homeostasis (65, 66), which affects numerous other cell signaling pathways.

Despite these critical metabolic roles of mitochondria, classic "mitochondriology" was considered a mature field as recently as 1990. However, several important observations have fueled a renaissance in mitochondrial research, including *1*) mitochondrial ROS are not just damaging by-products of respiration, but important for cell signaling (19, 23); *2*) mitochondrial release of factors such as cytochrome *c* is an important step in programmed cell death (100, 110, 112); *3*) nitric oxide (NO·) is an potent regulator of mitochondrial function (19, 23, 34); *4*) mitochondrial morphology is far from static, with the organelles being subject to fission, fusion, and intracellular movement on a rapid timescale (95, 218); and *5*) mitochondria actively orchestrate the spatiotemporal profiles of intracellular Ca^{2+}, under both physiological and pathological conditions (65, 66). Together these observations suggest an extensive regulatory role for mitochondria in both normal and pathological cell function.

The interplay between the conventional and novel roles of mitochondria has received little consideration, and an examination of recent mitochondrial science reveals several incompatibilities with classic bioenergetic viewpoints. An example is the requirement of ATP for apoptosis (137). How does the cell maintain ATP synthesis in the face of mitochondrial disassembly that occurs during apoptosis? Another example, which is the focus of this review, is the role of Ca^{2+} in regulating organelle function and dysfunction. How can Ca^{2+}, a physiological stimulus for ATP synthesis (5, 72, 118), become a pathological stimulus for ROS generation, cytochrome *c* release, and apoptosis? As will be discussed extensively, this apparent mitochondrial Ca^{2+} paradox revolves around a "two-hit" hypothesis (Fig. 1) in which a concurrent pathological stimulus can turn Ca^{2+} from a physiological to a pathological effector.

MITOCHONDRIAL Ca^{2+} UPTAKE AND RELEASE PATHWAYS

Most of the mitochondrial effects of Ca^{2+} require its entry across the double membrane into the matrix. Although the mitochondrial outer membrane was thought to be permeable to

Address for reprint requests and other correspondence: S.-S. Sheu, Univ. of Rochester Medical Center, 601 Elmwood Ave., Box 711, Rochester, NY 14642 (E-mail: sheyshing_sheu@urmc.rochester.edu).

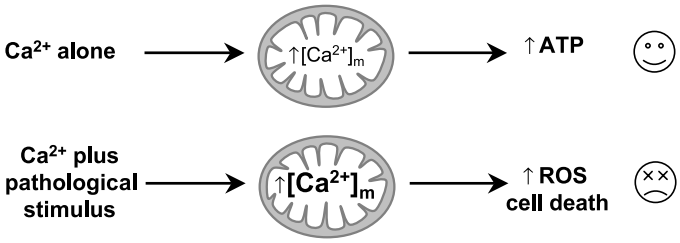

Fig. 1. Two-hit hypothesis for mitochondrial Ca^{2+} in physiology and pathology. Under physiological conditions, Ca^{2+} is beneficial for mitochondrial function. However, in the presence of an overriding pathological stimulus, Ca^{2+} is detrimental. Similarly, Ca^{2+} can potentiate a subthreshold pathological stimulus, resulting in pathogenic consequences. See text for full explanation. $[Ca^{2+}]_m$, mitochondrial matrix Ca^{2+} concentration; ROS, reactive oxygen species.

Ca^{2+}, recent studies suggest that the outer membrane voltage-dependent anion channel (VDAC) is a ruthenium red (RuRed)-sensitive Ca^{2+} channel and thus serves to regulate Ca^{2+} entry to mitochondrial intermembrane space (60). Furthermore, transport across the inner membrane is highly regulated (for review see Ref. 65).

Figure 2 outlines the major mechanisms for mitochondrial Ca^{2+} transport, with Ca^{2+} uptake achieved primarily via the mitochondrial Ca^{2+} uniporter (MCU). Uptake is driven by the membrane potential ($\Delta\psi_m$), and therefore the net movement of charge due to Ca^{2+} uptake consumes $\Delta\psi_m$. A recent patch-clamp study suggests the that MCU is a highly selective ($K_d <$ 2 nM) Ca^{2+} channel (99), but attempts to define its molecular nature have been largely unsuccessful. The channel is known mostly for its pharmacological sensitivity to RuRed (127), and a colorless component of RuRed (Ru360) is the active MCU-binding agent (156, 216). Saris et al. (165) identified a 40-kDa glycoprotein of the intermembrane space as an MCU regulatory component, although the transmembrane component of the MCU has been more difficult to isolate, with limited reports of such an entity (124). Interestingly, reverse MCU transport (Ca^{2+} export) was shown to be regulated by Ca^{2+} binding to the outer surface of the inner membrane (86) and was also linked to a soluble intermembrane space component.

Das et al. (41) showed that a complex of Ca^{2+}-polyphosphate and β-hydroxybutanoate can form a Ca^{2+} channel indistinguishable from that in *Escherichia coli*, raising the possibility that the MCU (by virtue of mitochondrial/bacterial relationships) may be a nonproteinaceous entity. However, the second-order Ca^{2+} transport kinetics of the MCU suggest a more complex structure with separate activation and transport sites (169, 206). From a physiological perspective, a role was recently demonstrated for p38 MAP kinase in regulating RuRed-sensitive Ca^{2+} transport (126). Clearly, identification of the molecular nature of the MCU will aid greatly in understanding the physiological and pathological regulation of mitochondrial Ca^{2+} uptake.

Two additional mechanisms of Ca^{2+} entry into mitochondria have also been identified. The first, called "rapid-mode" uptake (RaM), occurs on a millisecond timescale and allows fast changes in mitochondrial matrix Ca^{2+} concentration ($[Ca^{2+}]_m$) to mirror changes in the cytosol ($[Ca^{2+}]_c$) (186). Second, we have found (11) that ryanodine receptor isoform (RyR)1 is localized to the inner membrane of mitochondria in excitable cells and have termed this channel "mRyR." Kinetic analysis of the MCU predicts a tetrameric structure like RyR, which exists as a tetramer of ~500-kDa subunits (17). Together, mRyR and RaM are thought to underlie the phenomenon of excitation-metabolism coupling, in which $[Ca^{2+}]_c$-induced contraction is matched by $[Ca^{2+}]_m$ stimulation of ox-phos (see below).

A fast response of $[Ca^{2+}]_m$ to $[Ca^{2+}]_c$ requires rapid Ca^{2+} efflux from the mitochondrial matrix, and several mechanisms exist for this purpose (65). Primarily, Ca^{2+} efflux is achieved by exchange for Na^+, which is in turn pumped out of the matrix in exchange for protons (Fig. 2). Thus both Ca^{2+} uptake and efflux from mitochondria consume $\Delta\psi_m$ and are therefore reliant on H^+ pumping by the respiratory chain to maintain this driving force. In addition to these pathways of Ca^{2+} efflux, an additional mechanism exists in the form of the permeability transition (PT) pore (10). The PT pore is assembled from a group of preexisting proteins in the mitochondrial inner and outer membranes (38), with Ca^{2+} binding sites on the matrix side of the inner membrane believed to regulate pore activity. Normally, "flickering" of the PT pore between open and closed states serves to release Ca^{2+} from the matrix (84, 141, 205). However, prolonged PT pore opening due to $[Ca^{2+}]_m$ overload can result in pathological consequences (38).

Ca^{2+} AS POSITIVE EFFECTOR OF MITOCHONDRIAL FUNCTION.

The primary role of mitochondrial Ca^{2+} is the stimulation of ox-phos (5, 40, 72, 118, 123). As shown in Fig. 3, this occurs at many levels, including allosteric activation of pyruvate dehydrogenase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase (118), as well as stimulation of the ATP synthase (complex V) (40), α-glycerophosphate dehydrogenase (211), and the adenine nucleotide translocase (ANT) (123). Overall the effect of elevated $[Ca^{2+}]_m$ is the coordinated upregulation of the entire ox-phos machinery, resulting in faster respiratory chain activity and higher ATP output. Thus mitochondrial ATP output can be changed to meet the cellular ATP demand. An example of this is β-adrenergic stimulation

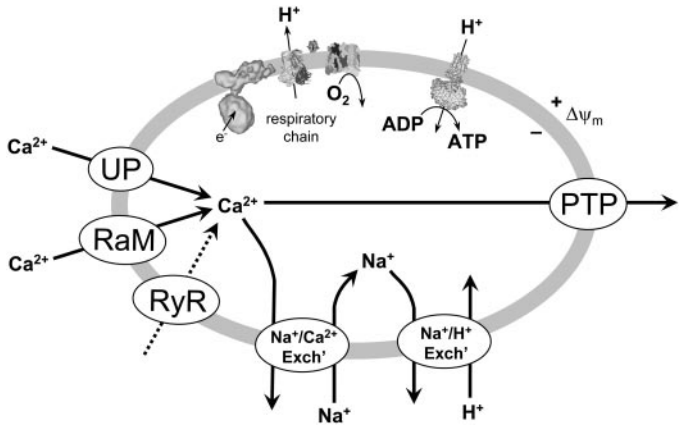

Fig. 2. Pathways of mitochondrial Ca^{2+} uptake and export. The respiratory chain is shown with (*left* to *right*) complexes I, III, IV, and V. The outer mitochondrial membrane and complex II are omitted for clarity. Where possible, known 3-dimensional (3D) structures obtained from the Protein Data Bank (**http://www.rcsb.org/pdb**) are shown. UP, Ca^{2+} uniporter; RaM, rapid-mode Ca^{2+} uptake; RyR, ryanodine receptor; PTP, permeability transition pore; $\Delta\psi_m$, membrane potential.

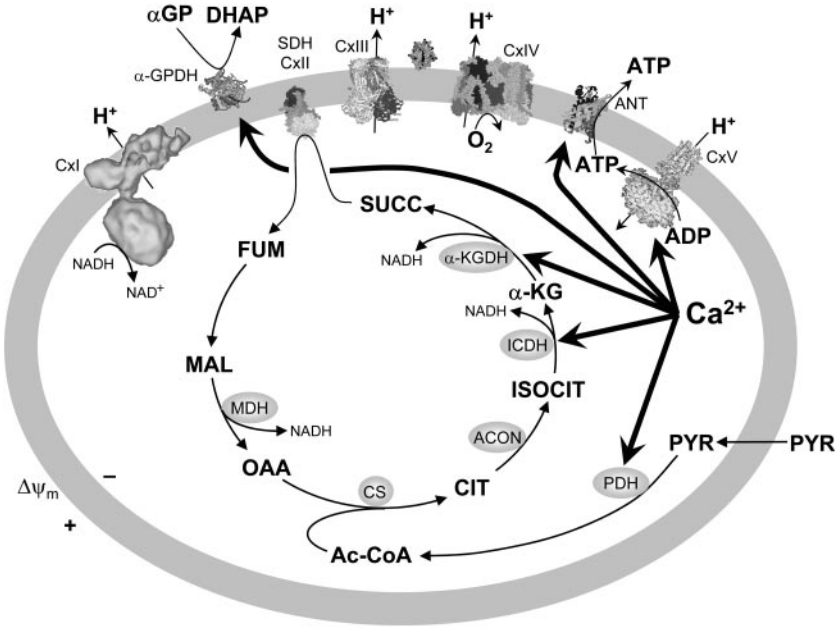

Fig. 3. Ca^{2+} activation of the TCA cycle and oxidative phosphorylation. Thin arrows represent metabolic pathways/reactions; thick arrows represent actions of Ca^{2+}. The outer membrane is omitted for clarity. Where possible, known 3D structures obtained from the Protein Data Bank are shown. For α-glycerophosphate (α-GP) dehydrogenase (α-GPDH), the cytosolic isoform structure is shown. Succ, succinate; α-KG, α-ketoglutarate; Isocit, isocitrate; Cit, citrate; OAA, oxaloacetate; Mal, malate; Fum, fumarate; Ac-CoA, acetyl coenzyme A; Pyr, pyruvate; PDH, pyruvate dehydrogenase; Acon, aconitase; CS, citrate synthase; MDH, malate dehydrogenase; ICDH, isocitrate dehydrogenase; α-KGDH, α-ketoglutarate dehydrogenase; DHAP, dihydroxyacetone phosphate; CxI–V, complexes I–V; SDH, succinate dehydrogenase.

in cardiomyocytes signaling the demand for increased contractility. The concomitant upregulation of ox-phos via $[Ca^{2+}]_m$ elevation provides the ATP needed for increased contractile force.

Many other mitochondrial functions are also regulated by Ca^{2+}. For example, Ca^{2+} activation of *N*-acetylglutamine synthetase generates *N*-acetylglutamine (92), a potent allosteric activator of carbamoyl-phosphate synthetase, the rate-limiting enzyme in the urea cycle (119). In addition, Ca^{2+}- and diacylglycerol-sensitive protein kinase (PKC) isoforms and calmodulin have been reported in mitochondria, although their precise targets within the organelle are less well understood (54, 160).

Overall, it appears that Ca^{2+} is a global positive effector of mitochondrial function, and thus any perturbation in mitochondrial or cytosolic Ca^{2+} homeostasis will have profound implications for cell function, for example, at the level of ATP synthesis. Also, it cannot be ignored that Ca^{2+}, particularly at the high concentrations experienced in pathology, appears to have several negative effects on mitochondrial function, as discussed in the following sections.

Ca^{2+} OVERLOAD AND PERMEABILITY TRANSITION PORE

In contrast to the beneficial effects of Ca^{2+}, the PT pore embodies the pathological effects of Ca^{2+} on mitochondria. The PT pore, as described nearly 25 years ago by Haworth and Hunter (81–83), is an assembly of preexisting proteins of the inner and outer mitochondrial membranes into a large conductance channel permeable to solutes of <1,500 Da. Debate still surrounds the composition of the PT pore, and although a full discussion is beyond the scope of this review, Fig. 4 shows the key components. Along with VDAC, ANT, and cyclophilin D (Cyp-D), several proteins are believed to regulate the pore (for review see Ref. 38).

The PT pore is triggered by high $[Ca^{2+}]_m$ and other stimuli including oxidants and the depletion of adenine nucleotides. It is inhibited by acidic pH, antioxidants such as reduced glutathione (GSH), and cyclosporin A, which binds to Cyp-D, a matrix *cis*/*trans*-prolyl-isomerase. The ANT appears to be a key modulator, with ANT thiols proposed as a target for oxidative stress induction of the PT pore (69). Also, bongkre-

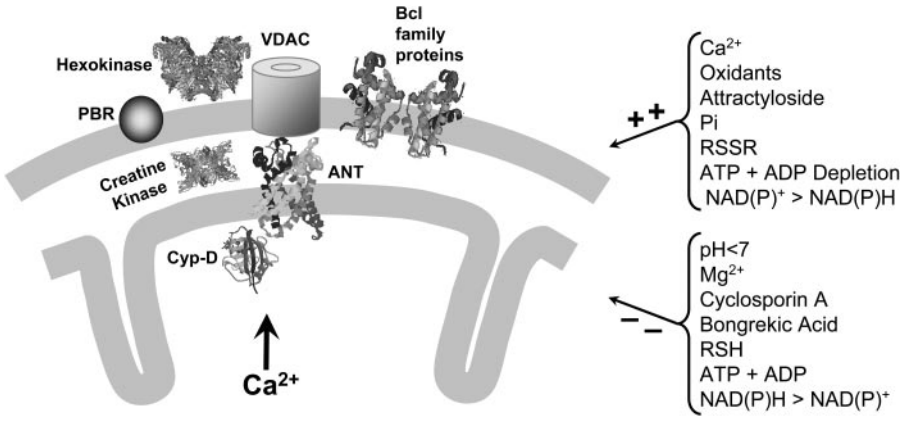

Fig. 4. The mitochondrial permeability transition pore. The putative components of the pore are shown, although the exact arrangement and stoichiometry are not known. Where possible, known 3D structures from the Protein Data Base are shown. In the case of cyclophilin D (Cyp-D), the structure of Cyp-A is shown. ANT, adenine nucleotide translocase; PBR, peripheral benzodiazepine receptor; VDAC, voltage-dependent anion channel; RSH, reduced thiol; RSSR, thiol disulfide.

kic acid and attractyloside, two inhibitors of ANT, inhibit or activate PT pore opening, respectively. Recently, doubt has been cast on the role of ANT, because mitochondria isolated from double-knockout $ANT1^{-/-}$, $ANT2^{-/-}$ mice appear capable of undergoing PT (101). However, the ANT is a mitochondrial carrier family protein, all of which share significant sequence homology, making it likely that other proteins in this family can substitute for the ANT in PT pore assembly.

The role of the PT pore in pathological cell injury and death has been cemented by the discovery that opening of this pore is mechanistically linked to cytochrome *c* release, a key event in apoptosis (112). Despite the recent popularity of this research topic, it is worth noting that Knyazeva et al. (100) discovered mitochondrial cytochrome *c* release in ischemic liver nearly 30 years ago! Several studies suggest a role for the PT pore in this process, including findings that *1*) PT pore inhibitors (e.g., cyclosporin A) inhibit cytochrome *c* release and apoptosis (176, 222), *2*) the Bcl family proteins have been shown to functionally interact with PT pore components such as VDAC (132, 177, 200), and *3*) the loss of $\Delta\psi_m$ is a hallmark of apoptotic cell death and is thought to signal the recruitment of Bcl family proteins to the mitochondrion (44).

Despite strong evidence linking the PT pore, cytochrome *c* release, and apoptosis, the precise mechanism of cytochrome *c* release is still unknown and is likely to be dependent on cell type, apoptotic stimulus, and precise cellular conditions. Several non-PT pore-mediated mechanisms of cytochrome *c* release may exist, and it is important to emphasize that cytochrome *c* does not exit through the PT pore itself. Also, although in vitro PT pore opening results in mitochondrial swelling and outer membrane rupture (38, 143), this is unlikely to occur in vivo, because mitochondrial swelling is not typically observed in apoptosis (although it is in necrosis) (111). This is in agreement with our data from time-course experiments in isolated mitochondria showing that cytochrome *c* release is temporally unrelated to swelling (24). Overall, the PT pore can be considered an important signaling pathway leading to cytochrome *c* release, but its involvement in the physical mechanism of cytochrome *c* release is still debated.

Cytochrome *c* is highly positively charged and binds to negatively charged cardiolipin on the outside of the inner membrane. There are also binding sites on respiratory complexes III and IV, and it has been shown that cytochrome *c* release is a two-step process (143), involving release of the protein from its inner membrane binding sites followed by outer membrane translocation. In addition, PT pore opening appears to be accompanied by a burst of ROS (61, 62), and this phenomenon is proposed to be involved in the autoamplification phase of the pore (62, 102). Because ROS can cause oxidation of cardiolipin, changing its physical properties (204), this may also enhance cytochrome *c* release (62, 143). Therefore, it is possible that high $[Ca^{2+}]$ in the intermembrane space may enhance cytochrome *c* release by competing it off binding sites (89), through a mechanism involving ROS oxidation of cardiolipin. Within the overall context of Ca^{2+} as a mitochondrial pathological stimulus, we have shown (21) that PT pore triggering by ROS is potentiated by Ca^{2+}. This is an example of the two-hit hypothesis (Fig. 1), in which the combination of Ca^{2+} plus a pathological stimulus such as ROS can elicit mitochondrial dysfunction.

In addition, recent evidence has suggested that cytochrome *c* can bind to the endoplasmic reticulum (ER) inositol 1,4,5-trisphosphate receptor (IP_3R), rendering the channel insensitive to autoinhibition by high $[Ca^{2+}]_c$ and resulting in enhanced ER Ca^{2+} release (14, 15). Thus Ca^{2+}-induced mitochondrial cytochrome *c* release may propagate apoptotic signaling by promoting further Ca^{2+} overload. The close proximity between the ER and mitochondria (115) facilitates this cross-talk and is discussed in the next section.

MITOCHONDRIAL MORPHOLOGY, DYNAMICS, AND ER COORDINATION

Morphological alterations of mitochondria have been observed in multiple pathologies, implying a close relationship between mitochondrial structure and function. However, even in normal cells (95) mitochondria display dynamic tubular networks undergoing constant fission, fusion, and cytoskeletal trafficking (8, 9, 138). These networks appear to intertwine closely with tubules of the ER. Although the reason for dynamic mitochondrial behavior is not fully understood, it is believed to ensure appropriate mitochondrial distribution to provide ATP to localized cytosolic regions.

Mitochondrial fission requires the dynamin-like protein DLP1, a large GTPase that is transiently recruited to mitochondria by interactions with the outer membrane protein hFis1 (13, 142, 151, 179, 180, 217–219). Fusion is also mediated by GTPases; mitofusin (Mfn) is an outer membrane anchored GTPase and a homolog of the *Drosophila* "fuzzy onion" protein (31, 67, 85, 104, 158, 163, 164). Two mammalian Mfn isoforms (Mfn1 and Mfn2) have both redundant and distinct functions, forming homo- and heteromeric complexes (31). The coordinated fusion of inner and outer membranes is thought to occur when Fzo1p (a yeast homolog of fuzzy onion) forms a complex with Mgm1p, which is an inner membrane-associated dynamin family GTPase (174, 213).

Mitochondrial fission and fusion must occur at balanced levels to maintain normal morphology (173), and an imbalance results in excessive fragmentation or tubulation, with pathological consequences. Increased fission appears to be a prerequisite for cytochrome *c* release, because mitochondrial morphology in many apoptotic cells changes from tubular networks to a fragmented phenotype (95). For example, hFis1 overexpression causes mitochondrial fragmentation, cytochrome *c* release, and apoptosis (90, 217), whereas a dominant-negative DLP1 prevents cytochrome *c* release in staurosporine-induced apoptosis (57). Similarly, $Mfn^{-/-}$ mice die at midgestation (31), and the human homolog of Mgm1p has been identified as OPA1, which causes hereditary blindness when mutated (4, 46). Notably, DLP1 and Mfn2 have been shown to closely appose to proapoptotic Bax (94), and the development of mitochondrial fragmentation has been demonstrated in several apoptotic cell systems, indicating that the mitochondrial fission/fusion machinery directly or indirectly interacts with apoptotic components to regulate cell death (18).

Recent studies have suggested coordination between Ca^{2+} signaling of the ER and mitochondria, facilitated by strategic location of mitochondria at sites of ER Ca^{2+} release (115). The ER is the major intracellular Ca^{2+} store, and regulated Ca^{2+} release from this organelle is essential for cellular signaling. However, in pathological situations this may be detrimental to

mitochondrial function, and Ca^{2+} released during the ER stress response may promote mitochondrial fragmentation and apoptosis. As noted above, it has also been reported that cytochrome *c* can bind to the IP_3R, causing further ER Ca^{2+} release (14, 15). Because mitochondrial cytochrome *c* release during apoptosis is an "all-or-nothing" event occurring within a rapid time frame (116), it has been suggested that Ca^{2+} is the coordinating signal for cytochrome *c* release (15).

Several questions arise from these studies, such as the following: How can the cell avoid cytochrome *c* release during normal fission/fusion events? Is cytochrome *c* release in apoptosis merely an accidental by-product of mitochondrial fission (leakage?), or is fission a deliberate part of the mechanism of cytochrome *c* release? It is possible that when the distance between two fission sites is extremely close, the short lipid bilayer may not be able to provide sufficient curvature for membrane sealing, causing membrane rupture and leakage. In support of this notion, cytochrome *c* release on hFis1 overexpression is not inhibited by classic inhibitors of PT pore opening (90), suggesting that the PT pore is not involved in the mechanism of cytochrome *c* release under these conditions.

An additional link between ER Ca^{2+}, mitochondria, and apoptosis is provided by studies showing that overexpression of antiapoptotic Bcl-2 lowers the steady-state ER $[Ca^{2+}]$ ($[Ca^{2+}]_{ER}$), leading to lower mitochondrial Ca^{2+} uptake on IP_3R activation (56, 150). In addition, proapoptotic $Bax^{-/-}$-$Bak^{-/-}$ fibroblasts also display lower $[Ca^{2+}]_{ER}$ (172). These data suggest that increased ER Ca^{2+} release is a key event in apoptosis regulated by both pro- and antiapoptotic Bcl proteins. Furthermore, a direct role for ER Ca^{2+} in regulating mitochondrial fission has been proposed; ectopic expression of the p20 fragment of BAP31 (an ER protein that binds to Bcl-2 and Bcl-X_L) causes mitochondrial translocation of DLP1, resulting in fragmentation and cytochrome *c* release (18). The signal for mitochondrial recruitment of DLP1 is likely to be elevated $[Ca^{2+}]_m$, because inhibition of mitochondrial Ca^{2+} uptake attenuates the fragmentation (18).

In summary, coordination between ER and mitochondria occurs both spatiotemporally and biochemically and has the potential to regulate mitochondrial morphology. Perturbation of the ER can lead to $[Ca^{2+}]_m$ overload, fragmentation, and apoptosis. This is a further example of the two-hit hypothesis (Fig. 1), in which a normal mitochondrial process (fission/fusion) can have pathological consequences in the presence of high $[Ca^{2+}]$.

MITOCHONDRIAL ROS GENERATION

Mitochondria are a significant source of ROS, although the assumption that 1–2% of all O_2 consumed ends up as ROS is likely an overestimate. Although an extensive review of mitochondrial ROS generation is beyond the scope of this review (see Refs. 88 and 202), an outline of the major sources within the organelle serves to highlight that this is both a physiological and a pathological phenomenon and will aid in the understanding of its regulation by Ca^{2+}.

The primary ROS made by mitochondria is superoxide ($O_2^-\cdot$), which is converted to H_2O_2 either by spontaneous dismutation or by the enzyme superoxide dismutase (SOD). H_2O_2 can be further transformed to $OH\cdot$ in the presence of metal ions by Fenton chemistry, although metal chaperone proteins in the mitochondrial matrix (37, 106) likely prevent this from occurring in the organelle.

The main source of $O_2^-\cdot$ in mitochondria is the ubisemiquinone radical intermediate ($QH\cdot$), formed during the Q cycle at the Q_O site of complex III (128, 192, 201). Generation of ROS is accelerated by complex III inhibitors distal to this site (e.g., antimycin A), although evidence that inhibition further down the respiratory chain (e.g., at complex IV) can also elevate ROS is sparse (19). The majority of $O_2^-\cdot$ from Q_O is made facing the intermembrane space (192), leading to suggestions that $O_2^-\cdot$ may be released through VDAC into the cytosol (71). However, the intermembrane space contains both Cu/Zn-SOD (139) and ~20 mM cytochrome *c*, which can be reduced by $O_2^-\cdot$ and pass the electrons on to complex IV (114). Indeed, exogenous $O_2^-\cdot$ is an excellent substrate for generation of $\Delta\psi_m$ and ATP synthesis (114), and mitochondria have been championed as a significant cellular ROS sink (63, 147, 224). Some $HO_2\cdot$ is probably also made from Q_O at the intermembrane space, because the acidic dissociation constant (pK_a) of $O_2^-\cdot$ is ~4.8 (45). Uncharged $HO_2\cdot$ can pass through membranes but is highly reactive and therefore likely initiates membrane lipid oxidation (45). Furthermore, the spontaneous dismutation of $HO_2\cdot$ (to H_2O_2) is five to eight orders of magnitude faster than for $O_2^-\cdot$. Under certain conditions, it has been shown that some $O_2^-\cdot/HO_2\cdot$ from Q_O may reach the matrix space (128, 192), but Mn-SOD in this compartment would rapidly convert it to H_2O_2.

Complex I is also a source of ROS, although the mechanism of generation is less clear than for complex III. Rotenone and other distal complex I inhibitors can cause $O_2^-\cdot$ generation facing the matrix side of the inner membrane (201, 192, 108), where Mn-SOD would convert it to H_2O_2. Recent reports suggest that glutathionylation of complex I (197) or phosphorylation by PKA (145, 153) can elevate ROS generation, but the physiological or pathological significance of this is unclear. In vitro, electrons entering at complex II (succinate dehydrogenase) can flow backward through complex I to make ROS (201, 202). In vivo this would be prevented by forward electron flow through complex I from NADH, except under pathological conditions in which NADH is depleted (see below).

Another important regulator of mitochondrial ROS is $\Delta\psi_m$. The generation of ROS is exponentially dependent on $\Delta\psi_m$ (187), and both chemical uncouplers (e.g., 2,4-dinitrophenol) (140) and the novel uncoupling proteins (UCPs) (133) appear to decrease mitochondrial ROS generation in whole cells and organs, although in vitro experiments with isolated mitochondria have revealed opposing effects (27). Interestingly, as we originally hypothesized in 1998 (20), it has now been shown that ROS can stimulate mitochondrial uncoupling (51, 130) and that the processes of uncoupling and ROS generation exist in a feedback loop (20, 130, 125).

Our understanding of the roles of mitochondrially derived ROS has been transformed in recent years. Previously ROS were thought to be damaging by-products of respiration, responsible for oxidative damage and contributing to aging (for review see Ref. 75). However, recent evidence has shown that mitochondrially derived ROS are important for a multitude of cell signaling processes (23). Illustratively, cytosolic Cu/Zn-$SOD^{-/-}$ mice are viable, whereas mitochondrial Mn-$SOD^{-/-}$ mice are not (79). The fact that Cu/Zn-SOD cannot compensate for the loss of Mn-SOD (121) suggests a much more subtle,

compartmentalized role for these enzymes than mere damage limitation and ROS detoxification. Several recent studies have shown that perturbations in mitochondrial ROS generation can affect diverse redox signaling pathways such as the cell cycle (167), cell proliferation (98), apoptosis (108), metalloproteinase function (155), oxygen sensing (30), protein kinases (154, 155), phosphatases (152), and transcription factors (78).

Ca^{2+} AND MITOCHONDRIAL ROS

At the heart of understanding how Ca^{2+} can be both a physiological and a pathological effector of mitochondrial function is the issue of how Ca^{2+} modulates mitochondrial ROS generation. In this section, the theoretical and experimental considerations underlying this "mitochondrial Ca^{2+} paradox" are discussed. Because the QH· intermediate in the Q cycle is a significant source of ROS (vide supra), two related parameters that can regulate ROS generation are *1*) the effective concentration of QH·, which is increased when the distal respiratory chain is inhibited, and *2*) the frequency of QH· occurrence, which is increased when the respiratory chain turns over more quickly. Thus both stimulation and inhibition of mitochondria can result in enhanced ROS generation.

Figure 5 shows the theoretical mechanisms by which Ca^{2+} can enhance ROS generation. Stimulation of the TCA cycle and ox-phos by Ca^{2+} would enhance ROS output by making the whole mitochondrion work faster and consume more O_2. Indeed, mitochondrial ROS generation correlates well with metabolic rate (148, 184), suggesting that a faster metabolism simply results in more respiratory chain electron leakage. In addition, Ca^{2+} stimulation of nitric oxide synthase (NOS) (3) generates NO·, which inhibits complex IV (34), and this would enhance ROS generation at Q_O. Indeed, we hypothesized (19) that a major physiological role for NO· is the regulation of mitochondrial ROS output. Thus mitochondria can act as a "redox signaling box," converting an NO· signal into an ROS signal (19). This signaling axis operates within a physiological window of NO· concentrations, and disruption of this axis by pathological levels of NO· is detrimental to mitochondrial ATP synthesis and cell function (178). Furthermore, NO·, in conjunction with high $[Ca^{2+}]_m$, can inhibit mitochondrial complex I (91), and this is another example of the two-hit hypothesis (Fig. 1) because NO· and Ca^{2+} are not detrimental to complex I function. Although other inhibitors of complex I are known to enhance its ROS generation (108, 197), it is not known whether this is the case for NO· + Ca^{2+}. Furthermore, Ca^{2+} can enhance cytochrome *c* dislocation from the mitochondrial inner membrane, either by competing for cardiolipin binding sites or by inducing the PT pore, and this results in an effective block of the respiratory chain at complex III, which would enhance ROS generation (62, 143). Another possibility is that Ca^{2+} can perturb mitochondrial antioxidant status. We have observed (unpublished data) that mitochondrial GSH is released very early in Ca^{2+}-induced PT pore opening, suggesting that Ca^{2+}-exposed mitochondria may generate more ROS because of diminished GSH levels.

Despite the theoretical events depicted in Fig. 5, experimental observations on Ca^{2+} and mitochondrial ROS are diverse. Overall it appears that Ca^{2+} diminishes ROS from complexes I and III under normal conditions but enhances ROS when the complexes are inhibited. Differences in the literature seem to stem from nuances of the specific respiratory chain inhibitors used, the presence of uncouplers, the source of the mitochondria, and the respiratory state (i.e., state 4 vs. state 3).

Addition of Ca^{2+} to rat heart mitochondria with a complex III inhibitor (antimycin A) caused a sharp increase in ROS generation, similar to that observed with FCCP (27). Because Ca^{2+} uptake causes mild uncoupling of mitochondria ($\Delta\psi_m$ dissipation), some of its effects on ROS generation may be due to this uncoupling effect. The mechanism by which uncouplers enhance ROS generation is unclear (201) but may involve perturbation of the pH gradient (ΔpH) across the mitochondrial membrane, which would affect the topology of $HO_2\cdot/O_2^-\cdot$ generation (45).

In contrast, addition of Ca^{2+} to brain mitochondria in the presence of antimycin A did not stimulate ROS generation from complex III, but Ca^{2+} did elicit a complex I ROS generation when rotenone was present (185). Notably, this effect was reversible by addition of EGTA, suggesting that the PT pore (irreversible cytochrome *c* loss) is not involved and hinting at a reversible Ca^{2+} binding site that may modulate ROS generation. This is supported by data showing that in liver mitochondria with antimycin A the ability of Ca^{2+} to stimulate

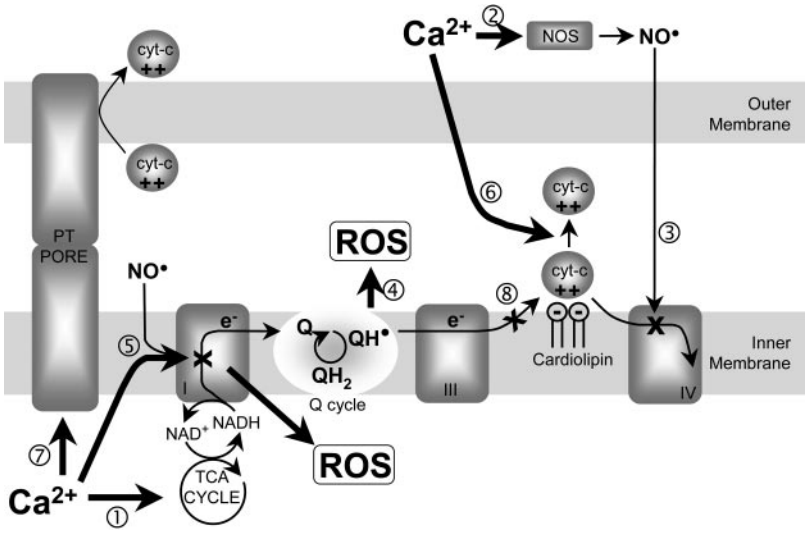

Fig. 5. Mechanisms for Ca^{2+} stimulation of mitochondrial ROS generation. Ca^{2+} stimulation of the TCA cycle (*1*) will enhance electron flow into the respiratory chain, and Ca^{2+} stimulation of nitric oxide synthase (NOS) and subsequent nitric oxide (NO·) generation (*2*) would inhibit respiration at complex IV (*3*). These events would enhance ROS generation from the Q cycle (*4*). In addition, NO· and Ca^{2+} can inhibit complex I, possibly enhancing ROS generation from this complex (*5*). Ca^{2+} also dissociates cytochrome *c* (cyt-c) from the inner membrane cardiolipin (*6*) and at high concentrations triggers PTP opening and cytochrome *c* release across the outer membrane (*7*). The subsequent inhibition at complex III (*8*) would enhance ROS generation at the Q cycle (*4*) Complex II is omitted from this diagram for clarity.

ROS generation is inhibited by the local anesthetic dibucaine and by Mg^{2+} (102). The authors attribute this effect to the ability of these species to displace Ca^{2+} from its membrane protein binding sites (102).

A further study on Ca^{2+} and brain mitochondria showed that in the presence of rotenone plus complex I substrates, Ca^{2+} decreased ROS generation (188). This was not due to uncoupling, because Ca^{2+}-induced loss of $\Delta\psi_m$ lasted only a few seconds whereas ROS generation was depressed for several minutes. With oligomycin present, Ca^{2+} caused large-scale uncoupling that lasted for several minutes. Notably, this was reversed by EGTA, again suggesting a reversible Ca^{2+} binding site that regulates ROS (188).

Overall these experiments suggest that in a tissue-specific manner, Ca^{2+} diminishes ROS from both complexes I and III under normal conditions and enhances ROS when these complexes are inhibited. The exact mechanism of Ca^{2+}-induced ROS generation is unclear, although it may involve changes in the three-dimensional conformation of the respiratory complexes. Indeed, Ca^{2+} is reported to alter the spectrum of cytochromes a/a_3 in isolated complex IV (212), and we have shown (21) that Ca^{2+} exposes novel mitochondrial targets for nitration by $ONOO^-$, consistent with protein conformational changes. Ultimately, differences in the subunit composition of the respiratory complexes between tissues may underlie the tissue specificity of Ca^{2+} effects on ROS generation.

Together, Ca^{2+}-mediated events may have additive effects on ROS generation. For example, Ca^{2+} stimulation of the TCA cycle would increase electron flux into the proximal respiratory chain while Ca^{2+}-induced cytochrome *c* release would simultaneously inhibit the distal respiratory chain. Although thus far our two-hit model (Fig. 1) has concentrated on the convergence of Ca^{2+} with other pathological stimuli, it should be recognized that Ca^{2+} itself is a pathological stimulus and in some cases Ca^{2+} is both the first and second "hit," i.e., Ca^{2+} can modulate its own pathological effects.

Ca^{2+} AND PT PORE OPENING—A CAUSE OR EFFECT OF ROS GENERATION?

Apoptosis is reportedly accompanied by a burst of ROS (29, 61, 62, 102), although the recent discovery that the ROS fluorescent probe dichlorofluorescin (DCF) can be oxidized directly by cytochrome *c* raises the possibility of experimental artifacts due to apoptotic cytochrome *c* release (25). Studies on isolated mitochondria with other ROS probes have provided more information on this scenario, showing that a complex interplay exists between PT pore opening and ROS generation in response to Ca^{2+} (29, 61, 102, 204). What is still unclear is whether ROS generation is merely a consequence of PT pore opening and cytochrome *c* release or is an integral part of the signaling machinery of PT pore opening. Although evidence abounds that exogenously added ROS can trigger the PT pore (21, 29, 144), it is unclear whether endogenously generated ROS can perform this function. In support of a role for endogenous ROS in the PT pore, antioxidants and thiol reagents such as DTT are able to inhibit Ca^{2+}-induced PT pore opening (38). Furthermore, Castilho et al. (29) report that Ca^{2+}-induced PT pore opening is sensitive to the concentration of O_2 (the substrate for $O_2^-\cdot$ generation) and is inhibited by catalase, suggesting a role for H_2O_2 generation.

Theoretically, PT pore-mediated cytochrome *c* release would enhance ROS generation by inhibiting complex III, but experimental evidence for this is limited. A key issue is how much cytochrome *c* is required to sustain respiration. Studies on isolated complex IV have shown that very low levels of cytochrome *c* (1 μM) can sustain activity (7), suggesting that with millimolar levels of cytochrome *c* in the intermembrane space (39, 171), significant loss would not result in respiratory inhibition. Indeed, it appears that mitochondrial electron transport and ATP synthesis are maintained during apoptosis (208), presumably to allow execution of the apoptotic program, which requires ATP (137). However, it is worth noting that cytochrome *c* is not the only factor lost from mitochondria on PT pore opening. Nearly 100 proteins are lost from the intermembrane space (146), as well as GSH and other matrix solutes. Any of these components (especially GSH) could be responsible for enhanced ROS generation during apoptosis. Another idea developed within this context is that cytochrome *c* is an antioxidant, because it is an effective scavenger of $O_2^-\cdot$ and is readily diffusible and recycled in the cell (147). This raises the possibility that cytochrome *c* release during Ca^{2+}-/ROS-mediated mitochondrial dysfunction may have evolved as a protective strategy to scavenge extramitochondrial ROS before they reach the organelle.

Overall, the interplay among Ca^{2+}, ROS, and ATP in both the life and the death of the cell is extremely complex. This interplay is illustrated in Fig. 6, showing that the roles of each player in this "love-hate triangle" are different depending on the physiological/pathological status of the cell.

CLOSING THE TRIANGLE—ATP/ROS/Ca^{2+}

Thus far we have concentrated on the effects of Ca^{2+} on mitochondrial ROS generation and ATP, but it is important to briefly mention reverse signaling, i.e., the effects of ROS and ATP on Ca^{2+} homeostasis. A key example is the RyR, which has a critical thiol residue that can be modified by ROS (53) or reactive nitrogen species (RNS) (193, 214), thereby activating the channel and enhancing sarcoplasmic reticulum (SR) Ca^{2+} release. Thus mitochondrial ROS can theoretically modulate extramitochondrial Ca^{2+} pools. Consistent with these biochemical findings, it is reported that both SR and mitochondrial Ca^{2+} accumulation are inhibited by ROS (77, 93). Notably, at very high Ca^{2+} levels only SR Ca^{2+} uptake was inhibited, suggesting that in the face of a Ca^{2+} overload mitochondria continue to accumulate Ca^{2+}, which may contribute to downstream pathology (93). Notably, the inhibition of mitochondrial Ca^{2+} uptake by ROS was different depending on the respiratory substrate and was almost absent when mitochondria were incubated with a broad mix of substrates supporting complexes I, II, and IV. This suggests that oxidative damage to the respiratory chain may depolarize the mitochondrial inner membrane, impairing Ca^{2+} uptake, and is consistent with our observations showing that $ONOO^-$ can uncouple mitochondria (22). Such uncoupling prevents Ca^{2+} accumulation by diminishing the magnitude of $\Delta\psi_m$. The effects of NO· on mitochondrial Ca^{2+} have also been examined by several authors including ourselves (6, 24, 157); we (24) reported that NO· inhibition of mitochondrial respiration inhibits Ca^{2+} uptake by the organelle. It is tempting to speculate that under certain conditions Ca^{2+} activation of NOS, making NO·, may inhibit respiration

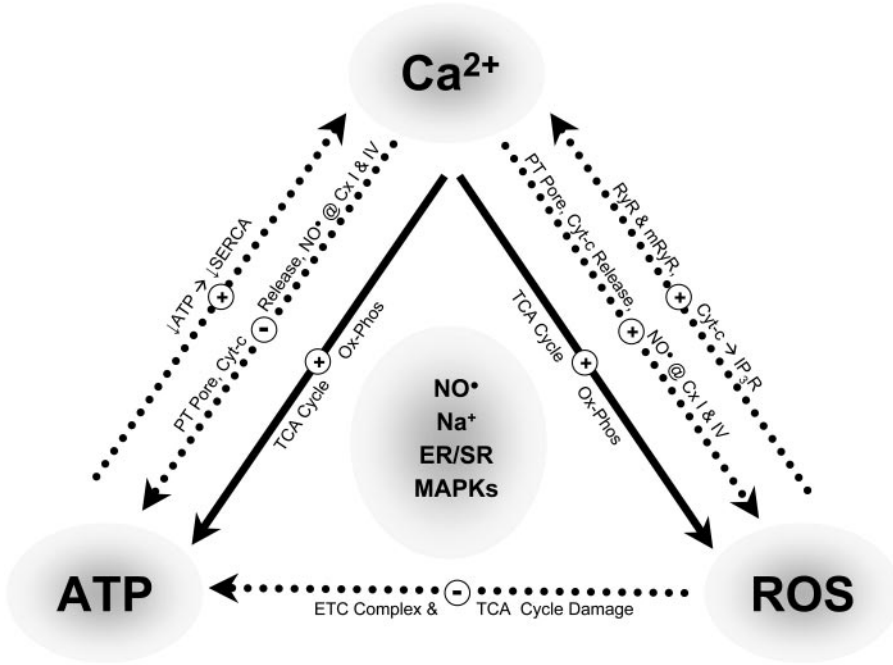

Fig. 6. The mitochondrial Ca^{2+}/ATP/ROS triangle. Solid arrows denote physiological effects; dotted arrows denote pathological effects. Words alongside the arrows describe specific events mediating the positive or negative effect. At the center of the triangle are factors that can orchestrate these effects. For full details, see text. SERCA, sarco(endo)plasmic reticulum Ca^{2+}-ATPase; ox-phos, oxidative phosphorylation; ER, endoplasmic reticulum; SR, sarcoplasmic reticulum; mRyR, mitochondrial RyR; IP_3R, inositol 1,4,5-trisphosphate.

as a protective mechanism to prevent mitochondrial Ca^{2+} accumulation.

A further level of cross-talk is suggested by Zoccarato et al. (224), who report that mitochondrial scavenging of external H_2O_2 is inhibited by Ca^{2+}. They attribute this to a Ca^{2+}-induced inactivation of glutathione reductase (GR) and glutathione peroxidase (GPX). The authors speculate that succinate-driven reverse electron flow through complex I, generating NADH (the substrate for GR and GPX), may underlie this phenomenon (224).

Finally, the importance of ATP in Ca^{2+} homeostasis cannot be understated, because the maintenance of ion gradients in all cell types depends on ATP (26). Therefore, any disturbance in ATP levels will have a large impact on SR, ER, and plasma membrane (PM) Ca^{2+} pumps. Overall, as highlighted in Fig. 6, it is clear that ATP, ROS, and Ca^{2+} exist in a triangular network, with each having the ability to control the others. The following sections discuss two pathological examples of this triangle in action.

EXAMPLES OF PATHOLOGICAL $[Ca^{2+}]_M$ OVERLOAD—CARDIAC ISCHEMIA-REPERFUSION INJURY

Overload of $[Ca^{2+}]_m$ is a key event in cardiac ischemia-reperfusion (I/R) injury (189, 190), with the end point being opening of the PT pore, cytochrome *c* release, and apoptosis/necrosis (38, 68). Figure 7 outlines the events preceding $[Ca^{2+}]_m$ overload. Under normal conditions, the PM Na^+/H^+ exchanger (NHE) equilibrates internal and external Na^+ and H^+ and the PM Na^+/Ca^{2+} exchanger keeps $[Ca^{2+}]_c$ low. During ischemia, lactic acidosis forces the NHE to import external Na^+, resulting in cytosolic Na^+ overload (96). Subsequently, the PM Na^+/Ca^{2+} exchanger is forced into reverse mode to dispose of excess Na^+, resulting in $[Ca^{2+}]_c$ overload. This Ca^{2+} is then taken up by mitochondria, resulting in $[Ca^{2+}]_m$ overload and all its consequences as described above (for reviews see Refs. 38 and 68).

Several levels of communication among Ca^{2+}, ROS, and ATP exist in this system, and at the center are the MAP kinases (ERK, JNK, p38). Mitochondrial ROS can activate MAP kinases (107, 154), and all three MAP kinases are activated during ischemia and, to a greater extent, on reperfusion (131, 221). Recently it was reported that mitochondria are the sole source of ROS for p38 activation in cardiomyocyte ischemia (103). Notably, ERK1/2 activation leads to phosphorylation and activation of NHE-1 (159, 183), and this may contribute to a feed-forward activation loop ($Ca^{2+} \rightarrow$ ROS $\rightarrow$ ERK $\rightarrow$ more $Na^+ \rightarrow$ more Ca^{2+}), enhancing $[Ca^{2+}]_m$ overload in I/R injury (Fig. 7*B*). Additionally, ERK signaling is critically dependent on mitochondrially generated ATP, which is unavailable during ischemia (1). This raises the important point that ATP is a substrate for all signaling kinases and therefore mitochondrial ATP synthesis has the potential to impact on cell signaling at multiple levels, not just MAP kinases.

In support of a role for MAP kinases in the cross-talk between ROS and Ca^{2+}, JNK has been shown to be elevated within mitochondria during I/R (77), raising the possibility of direct phosphorylation of mitochondrial respiratory complexes, with subsequent effects on ROS generation. In addition, p38 can inhibit the mitochondrial RuRed-sensitive Ca^{2+} uniporter (126), suggesting a protective role for this MAP kinase. However, in neurons, p38 can mediate the translocation of proapoptotic BH_3-containing proteins to mitochondria (59), suggesting opposing roles for p38 in different cell types.

Mitochondrial Ca^{2+} overload does not have to result in PT pore opening and cytochrome *c* release to be detrimental to heart function. For example, the inhibition of complex I that occurs in I/R injury is dependent on $[Ca^{2+}]_m$, because it is prevented by RuRed (73, 74). Several other mitochondrial enzymes are inhibited in response to I/R or ROS exposure, including aconitase, α-ketoglutarate dehydrogenase, and complexes III and IV (105, 161). Because the heart is almost entirely dependent on mitochondrially generated ATP for its

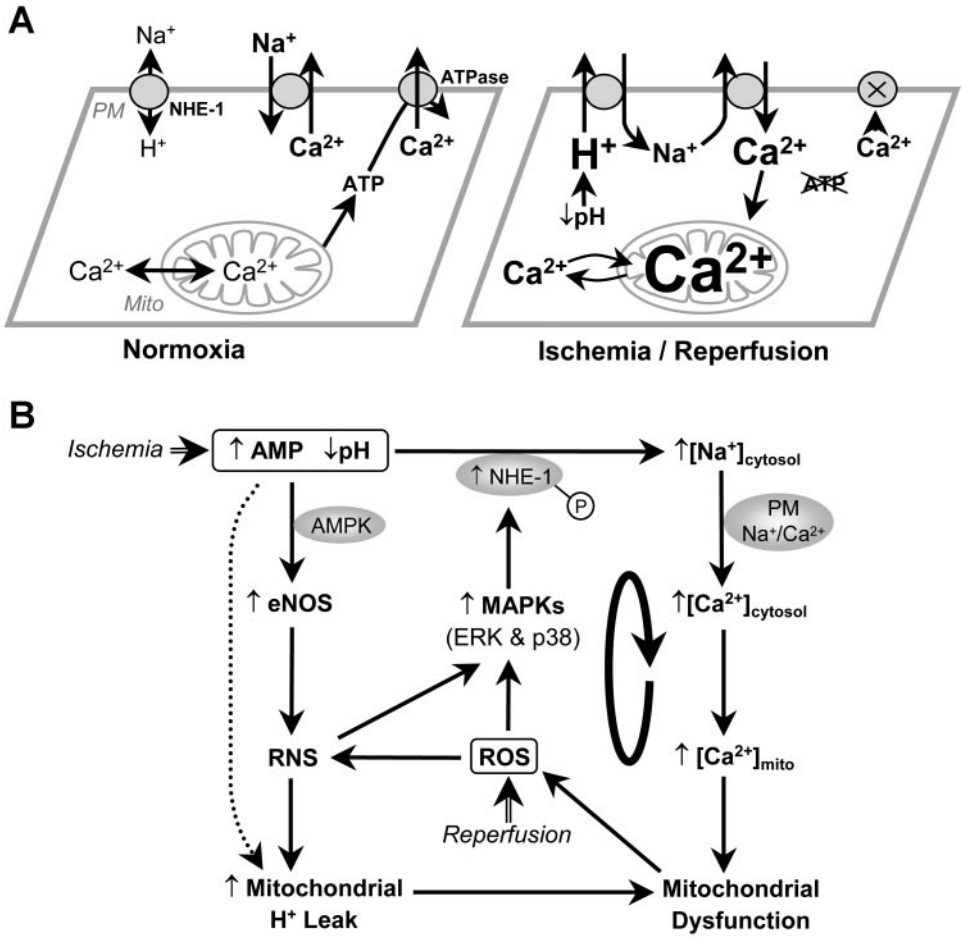

Fig. 7. Mitochondria, Ca^{2+}, and ROS in myocardial ischemia-reperfusion (I/R) injury. *A*: the events leading up to $[Ca^{2+}]_m$ overload during I/R. Briefly, a drop in pH triggers Na^+/H^+ exchanger (NHE)-1-mediated Na^+ influx, resulting in Na^+ overload. Reverse-mode Na^+/Ca^{2+} exchange across the plasma membrane (PM) then results in Ca^{2+} overload and subsequent $[Ca^{2+}]_m$ overload. Ca^{2+} cycling across the mitochondrial membrane can lead to propagation and amplification of $[Ca^{2+}]_m$ overload. In addition, ATP deficiency prevents adequate Ca^{2+} export by Ca^{2+}-ATPases. *B*: cross-talk between the pathways of mitochondrial dysfunction, ROS, and Ca^{2+} overload in I/R injury. The precipitating events (boxes) are acidosis, elevated AMP, and ROS generation. A feed-forward loop is hypothesized, encompassing mitochondrial ROS generation, MAPKs, and $[Ca^{2+}]_m$ overload. For further explanation, see text. RNS, reactive nitrogen species; AMPK, AMP-dependent protein kinase.

contractile energy, a defect at any level of the mitochondrial ox-phos machinery can have profound implications for contractile function on reperfusion.

Another important mitochondrial consequence of I/R injury is an increase in the H^+ leak (uncoupling) of the inner membrane (Fig. 7*B*; Ref. 16). This may involve the generation of RNS such as $ONOO^-$, which we demonstrated (22) can increase H^+ leak. In addition, $O_2^-\cdot$ and lipid oxidation products can activate mitochondrial UCPs (51, 125, 130), although this remains controversial (36). Alternatively, it has been shown that allosteric activation of the ANT by AMP turns it into a H^+ channel (28). Notably, AMP is elevated in I/R, and inhibitors of adenylate kinase (which catalyzes the reaction $2ADP \leftrightarrow ATP + AMP$) are of therapeutic benefit in cardiac I/R (81, 168). In addition, AMP-dependent protein kinase is known to activate endothelial NOS (eNOS) (3, 32), thereby leading to generation of NO· and possibly additional effects on mitochondria, MAP kinases, and other signaling pathways. Although at first glance mitochondrial uncoupling might be seen as detrimental to cardiac function because it would inhibit ATP generation, there may be benefits, too. Uncoupling would decrease $\Delta\psi$-dependent Ca^{2+} accumulation and would also decrease ROS generation. Indeed, several groups have demonstrated the efficacy of mitochondrial uncoupling (either chemical or by transfection of UCPs) as a protective strategy in I/R injury (12, 58, 117, 199). In summary, Fig. 7*B* outlines the roles of $[Ca^{2+}]_m$ overload, ROS generation, and diminished ATP in the progression of cardiac mitochondrial dysfunction in I/R.

EXAMPLES OF PATHOLOGICAL $[Ca^{2+}]_M$ OVERLOAD—NEURONAL EXCITOTOXICITY

Excitotoxicity is a phenomenon in which neuronal cells undergo necrosis or apoptosis in response to overexposure to excitatory amino acids such as glutamate (166). In particular, prolonged activation of the *N*-methyl-D-aspartate (NMDA) receptor leads to massive Ca^{2+} influx, resulting in $[Ca^{2+}]_c$ overload and cell death. Presently there are two schools of thought regarding the mechanisms of excitotoxicity: the mitochondrial Ca^{2+} hypothesis and the nuclear poly-ADP-ribose polymerase (PARP) hypothesis, with both theories emphasizing a key role for ROS.

The mitochondrial Ca^{2+} hypothesis of excitotoxicity centers on $[Ca^{2+}]_m$ overload as a central event in cell death (50, 135). In support of this, inhibition of $[Ca^{2+}]_m$ accumulation with the protonophore FCCP prevents excitotoxic cell death (191). Moreover, during the progression to excitotoxic cell death, a late secondary increase in $[Ca^{2+}]_c$ occurs, termed "delayed Ca^{2+} deregulation" (DCD) (134, 135). Although the mechanism of DCD is still under debate, it is agreed that the speed of DCD is strongly dependent on the magnitude of $[Ca^{2+}]_m$

accumulation, suggesting that mitochondrial Ca^{2+} release causes DCD (134).

Parallel to the mitochondrial Ca^{2+} hypothesis, a role for PARP-1 in excitotoxicity has been established by showing that PARP-1 inhibitors or genetic knockouts prevent excitotoxicity (149, 194). The mechanism of PARP-1-mediated excitotoxicity has been proposed to involve NMDA-receptor-mediated Ca^{2+} influx, leading to activation of neuronal NOS (nNOS) and excessive production of NO· (43). Overload of $[Ca^{2+}]_c$ also activates the protease calpain (33), which converts xanthine dehydrogenase (XDH) to xanthine oxidase (XO), leading to ROS generation. Interestingly, mitochondria also contain a protease that can convert XDH to XO, and this enzyme is released under conditions of mitochondrial stress (162). Subsequently, the generation of $ONOO^-$ (from NO· and O_2^-·) damages DNA and both directly and indirectly activates PARP-1 (149, 194, 207). PARP-1 then catalyzes the hydrolysis of NAD^+ into nicotinamide plus PAR, causing depletion of NAD^+ (2). This results in cellular energy failure and necrotic cell death. Intriguingly, it has been shown that PARP-1 activation, possibly via generation of non-protein-bound PAR polymers, leads to a loss of $\Delta\psi_m$ and the release of both cytochrome *c* and apoptosis-inducing factor (AIF) (220). In addition, NO· has been shown to elicit synaptic glutamate release (120) and may therefore contribute to further excitotoxicity in neighboring cells.

The interplay between PARP-1 and Ca^{2+} in excitotoxicity is shown in Fig. 8 and demonstrates the central role of mitochondria. The first major signal on NMDA receptor activation is an overload of $[Ca^{2+}]_c$, which leads to generation of ROS/RNS. However, the amount of ROS/RNS generated via cytosolic Ca^{2+}-dependent pathways appears to be insufficient for cell death, because blockade of $[Ca^{2+}]_m$ uptake averts excitotoxicity (191). Therefore, Ca^{2+} stimulation of mitochondrial ROS may also be important. In addition, $ONOO^-$ is a potent trigger of the PT pore and cytochrome *c* release in Ca^{2+} loaded mitochondria (21, 144) but cannot induce PT alone (i.e., without Ca^{2+}) (21). Thus it appears that the concerted effects of RNS and Ca^{2+} on mitochondria are required for cell death to occur—another demonstration of the two-hit hypothesis (Fig. 1).

Further interaction between PARP-1 and ROS could occur at the level of $NAD(P)^+$/NAD(P)H. Because GR requires NAD(P)H to reduce oxidized glutathione (GSSG) back to GSH, a depletion in $NAD(P)^+$ would lead to a depletion in the reducing substrates NAD(P)H and a subsequent shift in GSH-to-GSSG ratio. PT pore opening is dependent on the redox status of both the NAD(P)H and GSH pools, being greater in the oxidized state (38). In addition, $NAD(P)^+$ depletion may impact on mitochondrial ATP synthesis, and this has profound implications for a cell such as the neuron that relies heavily on mitochondrial ATP for the maintenance of PM ion gradients. Overall, it appears that $[Ca^{2+}]_m$ overload and subsequent mitochondrial dysfunction are unifying factors in excitotoxicity, playing key roles in cell death. As discussed in the next section, mitochondria are emerging as the target of choice for therapeutic intervention in neurodegenerative disease.

THERAPEUTIC STRATEGIES

Direct targeting of drugs to mitochondria has long been a dream of pharmacologists, but only recently has it been realized that multiple pharmacological approaches that are not necessarily directed at the mitochondrion appear to converge on $[Ca^{2+}]_m$ accumulation as an end point for cardio- and neuroprotection (129). An example is the NHE-1 inhibitor cariporide, which is entering stage 3 clinical trials and appears to work by preventing Na^+ overload and subsequent $[Ca^{2+}]_m$ overload (42, 48). Another example is ischemic preconditioning (IPC), in which a series of small ischemic events can

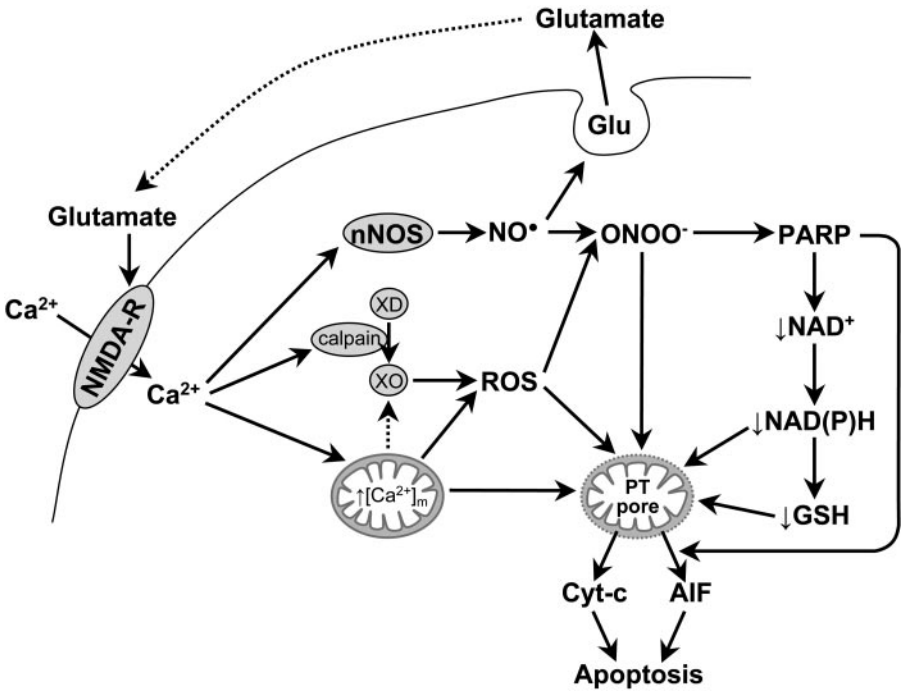

Fig. 8. Mitochondria, Ca^{2+}, and ROS in neuronal excitotoxicity. After *N*-methyl-D-aspartate receptor (NMDA-R)-mediated Ca^{2+} influx, the generation of both NO· and ROS elicits downstream cell death signaling. The relative importance of ROS from mitochondria and other sources is not fully understood. For further explanations, see text. nNOS, neuronal NOS; PARP, poly-ADP-ribose polymerase; GSH, reduced glutathione; AIF, apoptosis-inducing factor; XD, xanthine dehydrogenase; XO, xanthine oxidase.

protect against injury from a large ischemic event (215, 223). The sulfonylurea drugs (e.g., diazoxide) are proposed to work by mimicking the effects of IPC, opening a mitochondrial ATP-sensitive potassium (K^+_{ATP}) channel, inhibiting $[Ca^{2+}]_m$ uptake and subsequent PT pore opening (195). Interestingly, ROS (possibly mitochondrial) are implicated in IPC signaling (223). In a similar manner, uncoupling mitochondria by chemical uncouplers (58) or by transfection UCPs (12, 117, 199) is both neuro- and cardioprotective, and these therapies are proposed to work by preventing $[Ca^{2+}]_m$ accumulation, ROS generation, and PT pore opening. Downstream of $[Ca^{2+}]_m$ overload, several strategies are aimed at preventing mitochondrial PT pore opening and cytochrome *c* release, including the PT pore inhibitor cyclosporin A, which is FDA indicated for ischemic heart disease.

An additional benefit of therapeutically targeting mitochondria comes from the increased cell functionality that results. A cardiomyocyte that is alive but not contracting is of very little use to the parent organism, and in this regard targeting mitochondria to maintain their integrity not only prevents cell death but also facilitates ATP synthesis, which is especially important in cells that are highly ATP dependent (e.g., myocytes and neurons). Clearly, the development of mitochondrially targeted drugs is an important research direction, and a range of strategies have been exploited to target therapeutic agents to mitochondria (182, 196, 209, 210).

One group of mitochondrially targeted molecules are prodrugs, which are activated by mitochondrial enzymes. The mitochondrial GSH pool plays an important role in cytoprotection (122), but to specifically investigate this compounds that selectively decrease mitochondrial GSH are required. In this regard, we synthesized 3-hydroxy-4-pentenoic acid (Fig. 9, *1*), which is biotransformed solely by mitochondrial 3-hydroxybutyrate dehydrogenase to 3-oxo-4-pentenoic acid (Fig. 9, *2*). 3-Oxo-4-pentenoic acid then reacts with GSH to give *adduct 3* (Fig. 9) and thereby depletes mitochondrial GSH (175). 3-Hydroxy-4-pentenoic acid markedly potentiates *t*-BuOOH-induced cell death (175).

Another property employed for mitochondrial drug delivery is $\Delta\psi_m$ (150–220 mV, positive outside). Some positively charged "mitochondriotropic" compounds can cross the mitochondrial membranes and accumulate in the matrix (209). Examples are the triphenylphosphonium (TPP)-based compounds. 4-Iodobutyltriphenylphosphonium (Fig. 9, *4*) is a re-

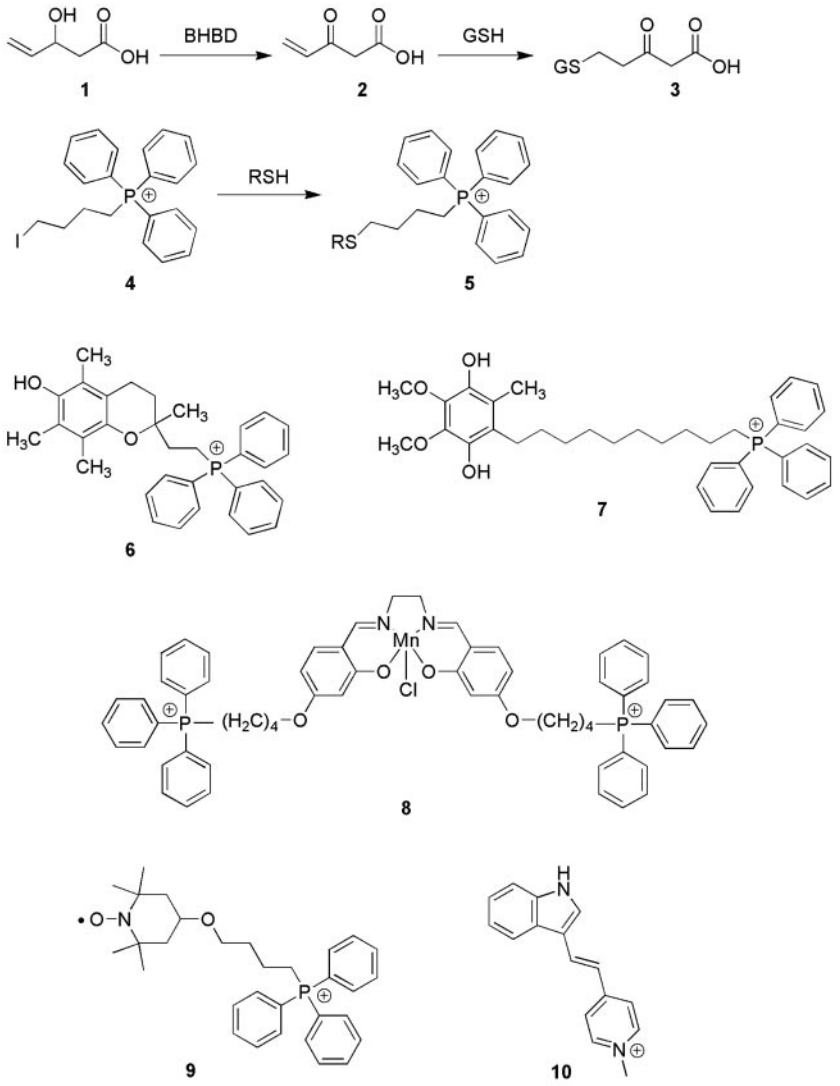

Fig. 9. Mitochondrially targeted compounds: *1*, 3-hydroxy-4-pentenoic acid; *2*, 3-oxo-4-hydroxypentenoic acid; *3*, 5-(glutathion-*S*-yl)-3-oxo-4-pentenoic acid; *4*, 4-iodobutyltriphenylphosphonium; *5*, sulfide analog of *4*; *6*, 2-[2-(triphenylphosphonio)ethyl]-3,4-dihydro-2,5,7,8-tetramethyl-2*H*-1-benzopyran-6-ol (Mito Vit E); *7*, 10-(6′-ubiquinolyl)decyltriphenylphosphonium (Mitoquinol); *8*, triphenylphosphonium cation conjugate of Salen-Mn(III) complex of *o*-vanillin (EUK-134); *9*, triphenylphosphonium cation conjugate of 4-hydroxy-2,2,6,6-tetramethylpiperidin-*N*-oxide (Tempol-TPP); *10*, 4-[2-(1*H*-indol-3-yl)-vinyl]-methylpyridinium (F16). BHBD, β-hydroxybutyrate dehydrogenase. RSH, protein thiol.

agent for selective labeling of thiols in mitochondria (35) and has been used to detect redox-dependent modification of mitochondrial proteins (Fig. 9, *5*; Ref. 109). Cationic lipophilic phosphonium salts derived from phosphonium chloride have also been prepared and tested (209).

To protect mitochondria from the pathological effects of ROS, several mitochondrially targeted antioxidants have been developed, including a TPP^+-linked vitamin E (Fig. 9, *6*) that protects mitochondria from iron/ascorbate and *t*-BuOOH-induced oxidative damage (181). A TPP^+-linked analog of ubiquinone (Fig. 9, *7*) has also been prepared (97). Interestingly, this compound protected cells from H_2O_2-induced apoptosis but not from staurosporine- or TNF-α-induced apoptosis.

Other studies have questioned the utility of using TPP^+ as a mitochondrial targeting strategy. Two TPP-based free radical scavengers, the TPP conjugate of Salen-Mn(III) complex of *o*-vanillin (EUK-134) (Fig. 9, *8*) and the TPP conjugate of Tempol (Fig. 9, *9*), delayed sodium selenite-induced apoptosis in HeLa cells but were no more effective than their nontargeted analogs (47). Clearly, more studies with a range of positively charged compounds are needed to determine the effectiveness of mitochondriotropics in combating mitochondrial dysfunction. In this regard, the compound 4-[2-(1*H*-indol-3-yl)-vinyl]-1-methylpyridinium (F16; Fig. 9, *10*) accumulates in mitochondria and selectively inhibits proliferation of a range of cancer cell lines (52).

In addition to the mitochondriotropics, a range of high-molecular-weight mitochondrially targeted compounds have been studied. These include peptide nucleic acid (PNA) oligomers, vesicular carriers, DQAsomes, cationic liposomes, and mitochondrial leader sequence peptide-oligonucleotide derivatives (209). The goal of targeting experimental tools and therapeutic agents to mitochondria will continue to be an active area of investigation, hopefully yielding therapies for diseases involving mitochondrial dysfunction.

CONCLUSIONS

Overall there appears to be a multifactorial cross-talk among Ca^{2+}, ATP, and ROS, centered on the mitochondrion (Fig. 6). Several of the key factors that mediate this cross-talk are also shown at the center of the triangle and include NO·, Na^+, MAP kinases, and functions of the ER and SR. Elucidating the molecular switches that control this complex triangle is already proving, and will continue to be, an exciting challenge.

ACKNOWLEDGMENTS

We thank Drs. Dieter Brdiczka and Thomas Gunter for comments on this manuscript. We also thank members of the University of Rochester Medical Center's Mitochondrial Research Interest Group for stimulating discussions.

GRANTS

This work is supported by National Institutes of Health Grants HL-71158-01 (to P. S. Brookes), DK-061991 (to Y. Yoon), and NS-37710 and HL-33333 (to S.-S. Sheu) and American Heart Association 0050839T and New York State Department of Health Spinal Cord Injury Research Fund C017688 to S.-S. Sheu.

REFERENCES

1. **Abas L, Bogoyevitch MA, and Guppy M.** Mitochondrial ATP production is necessary for activation of the extracellular-signal-regulated kinases during ischaemia/reperfusion in rat myocyte-derived H9c2 cells. *Biochem J* 349: 119–126, 2000.
2. **Alano CC, Ying W, and Swanson RA.** Poly(ADP-ribose) polymerase-1-mediated cell death in astrocytes requires NAD^+ depletion and mitochondrial permeability transition. *J Biol Chem* 279: 18895–18902, 2004.
3. **Alderton WK, Cooper CE, and Knowles RG.** Nitric oxide synthases: structure, function and inhibition. *Biochem J* 357: 593–615, 2001.
4. **Alexander C, Votruba M, Pesch UE, Thiselton DL, Mayer S, Moore A, Rodriguez M, Kellner U, Leo-Kottler B, Auburger G, Bhattacharya SS, and Wissinger B.** OPA1, encoding a dynamin-related GTPase, is mutated in autosomal dominant optic atrophy linked to chromosome 3q28. *Nat Genet* 26: 211–215, 2000.
5. **Balaban RS.** Cardiac energy metabolism homeostasis: role of cytosolic calcium. *J Mol Cell Cardiol* 34: 1259–1271, 2002.
6. **Balakirev MY, Khramtsov VV, and Zimmer G.** Modulation of the mitochondrial permeability transition by nitric oxide. *Eur J Biochem* 246: 710–718, 1997.
7. **Barone MC, Darley-Usmar VM, and Brookes PS.** Reversible inhibition of cytochrome *c* oxidase by peroxynitrite proceeds through ascorbate-dependent generation of nitric oxide. *J Biol Chem* 278: 27520–27524, 2003.
8. **Bereiter-Hahn J.** Behavior of mitochondria in the living cell. *Int Rev Cytol* 122: 1–63, 1990.
9. **Bereiter-Hahn J and Voth M.** Dynamics of mitochondria in living cells: shape changes, dislocation, fusion, and fission of mitochondria. *Microsc Res Tech* 27: 198–219, 1994.
10. **Bernardi P and Petronilli V.** The permeability transition pore as a mitochondrial calcium release channel: a critical appraisal. *J Bioenerg Biomembr* 28: 131–138, 1996.
11. **Beutner G, Sharma VK, Giovannucci DR, Yule DI, and Sheu SS.** Identification of a ryanodine receptor in rat heart mitochondria. *J Biol Chem* 276: 21482–21488, 2001.
12. **Bienengraeber M, Ozcan C, and Terzic A.** Stable transfection of UCP1 confers resistance to hypoxia/reoxygenation in a heart-derived cell line. *J Mol Cell Cardiol* 35: 861–865, 2003.
13. **Bleazard W, McCaffery JM, King EJ, Bale S, Mozdy A, Tieu Q, Nunnari J, and Shaw JM.** The dynamin-related GTPase Dnm1 regulates mitochondrial fission in yeast. *Nat Cell Biol* 1: 298–304, 1999.
14. **Boehning D, Patterson RL, Sedaghat L, Glebova NO, Kurosaki T, and Snyder SH.** Cytochrome *c* binds to inositol (1,4,5) trisphosphate receptors, amplifying calcium-dependent apoptosis. *Nat Cell Biol* 5: 1051–1061, 2003.
15. **Boehning D, Patterson RL, and Snyder SH.** Apoptosis and calcium: new roles for cytochrome *c* and inositol 1,4,5-trisphosphate. *Cell Cycle* 3: 252–254, 2004.
16. **Borutaite V, Mildaziene V, Brown GC, and Brand MD.** Control and kinetic analysis of ischemia-damaged heart mitochondria: which parts of the oxidative phosphorylation system are affected by ischemia? *Biochim Biophys Acta* 1272: 154–158, 1995.
17. **Bragadin M, Pozzan T, and Azzone GF.** Kinetics of Ca^{2+} carrier in rat liver mitochondria. *Biochemistry* 18: 5972–5978, 1979.
18. **Breckenridge DG, Stojanovic M, Marcellus RC, and Shore GC.** Caspase cleavage product of BAP31 induces mitochondrial fission through endoplasmic reticulum calcium signals, enhancing cytochrome *c* release to the cytosol. *J Cell Biol* 160: 1115–1127, 2003.
19. **Brookes P and Darley-Usmar VM.** Hypothesis: the mitochondrial NO· signaling pathway, and the transduction of nitrosative to oxidative cell signals: an alternative function for cytochrome *C* oxidase. *Free Radic Biol Med* 32: 370–374, 2002.
20. **Brookes PS.** Mitochondrial proton leak and superoxide generation: an hypothesis. *Biochem Soc Trans* 26: S331, 1998.
21. **Brookes PS and Darley-Usmar VM.** Role of calcium and superoxide dismutase in sensitizing mitochondria to peroxynitrite-induced permeability transition. *Am J Physiol Heart Circ Physiol* 286: H39–H46, 2004.
22. **Brookes PS, Land JM, Clark JB, and Heales SJ.** Peroxynitrite and brain mitochondria: evidence for increased proton leak. *J Neurochem* 70: 2195–2202, 1998.
23. **Brookes PS, Levonen AL, Shiva S, Sarti P, and Darley-Usmar VM.** Mitochondria: regulators of signal transduction by reactive oxygen and nitrogen species. *Free Radic Biol Med* 33: 755–764, 2002.
24. **Brookes PS, Salinas EP, Darley-Usmar K, Eiserich JP, Freeman BA, Darley-Usmar VM, and Anderson PG.** Concentration-dependent effects of nitric oxide on mitochondrial permeability transition and cytochrome *c* release. *J Biol Chem* 275: 20474–20479, 2000.
25. **Burkitt MJ and Wardman P.** Cytochrome *c* is a potent catalyst of dichlorofluorescin oxidation: implications for the role of reactive oxygen

species in apoptosis. *Biochem Biophys Res Commun* 282: 329–333, 2001.
26. **Buttgereit F and Brand MD.** A hierarchy of ATP-consuming processes in mammalian cells. *Biochem J* 312: 163–167, 1995.
27. **Cadenas E and Boveris A.** Enhancement of hydrogen peroxide formation by protophores and ionophores in antimycin-supplemented mitochondria. *Biochem J* 188: 31–37, 1980.
28. **Cadenas S, Buckingham JA, St-Pierre J, Dickinson K, Jones RB, and Brand MD.** AMP decreases the efficiency of skeletal-muscle mitochondria. *Biochem J* 351: 307–311, 2000.
29. **Castilho RF, Kowaltowski AJ, Meinicke AR, Bechara EJ, and Vercesi AE.** Permeabilization of the inner mitochondrial membrane by Ca^{2+} ions is stimulated by *t*-butyl hydroperoxide and mediated by reactive oxygen species generated by mitochondria. *Free Radic Biol Med* 18: 479–486, 1995.
30. **Chandel NS, McClintock DS, Feliciano CE, Wood TM, Melendez JA, Rodriguez AM, and Schumacker PT.** Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1α during hypoxia: a mechanism of O_2 sensing. *J Biol Chem* 275: 25130–25138, 2000.
31. **Chen H, Detmer SA, Ewald AJ, Griffin EE, Fraser SE, and Chan DC.** Mitofusins Mfn1 and Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development. *J Cell Biol* 160: 189–200, 2003.
32. **Chen ZP, Mitchelhill KI, Michell BJ, Stapleton D, Rodriguez-Crespo I, Witters LA, Power DA, Ortiz de Montellano PR, and Kemp BE.** AMP-activated protein kinase phosphorylation of endothelial NO synthase. *FEBS Lett* 443: 285–289, 1999.
33. **Cheng Y and Sun AY.** Oxidative mechanisms involved in kainate-induced cytotoxicity in cortical neurons. *Neurochem Res* 19:1557–1564, 1994.
34. **Cleeter MW, Cooper JM, Darley-Usmar VM, Moncada S, and Schapira AH.** Reversible inhibition of cytochrome *c* oxidase, the terminal enzyme of the mitochondrial respiratory chain, by nitric oxide. Implications for neurodegenerative diseases. *FEBS Lett* 345: 50–54, 1994.
35. **Coulter CV, Kelso GF, Lin TK, Smith RA, and Murphy MP.** Mitochondrially targeted antioxidants and thiol reagents. *Free Radic Biol Med* 28:1547–1554, 2000.
36. **Couplan E, del Mar Gonzalez-Barroso M, Alves-Guerra MC, Ricquier D, Goubern M, and Bouillaud F.** No evidence for a basal, retinoic, or superoxide-induced uncoupling activity of the uncoupling protein 2 present in spleen or lung mitochondria. *J Biol Chem* 277: 26268–26275, 2002.
37. **Craig EA and Marszalek J.** A specialized mitochondrial molecular chaperone system: a role in formation of Fe/S centers. *Cell Mol Life Sci* 59: 1658–1665, 2002.
38. **Crompton M.** The mitochondrial permeability transition pore and its role in cell death. *Biochem J* 341: 233–249, 1999.
39. **Darley-Usmar VM, Rickwood D, and Wilson MT.** *Mitochondria, A Practical Approach.* Oxford: IRL, 1987.
40. **Das AM and Harris DA.** Control of mitochondrial ATP synthase in heart cells: inactive to active transitions caused by beating or positive inotropic agents. *Cardiovasc Res* 24: 411–417, 1990.
41. **Das S, Lengweiler UD, Seebach D, and Reusch RN.** Proof for a nonproteinaceous calcium-selective channel in *Escherichia coli* by total synthesis from (*R*)-3-hydroxybutanoic acid and inorganic polyphosphate. *Proc Natl Acad Sci USA* 94: 9075–9079, 1997.
42. **Davies JE, Digerness SB, Goldberg SP, Killingsworth CR, Katholi CR, Brookes PS, and Holman WL.** Intra-myocyte ion homeostasis during ischemia-reperfusion injury: effects of pharmacologic preconditioning and controlled reperfusion. *Ann Thorac Surg* 76: 1252–1258, 2003.
43. **Dawson VL, Dawson TM, London ED, Bredt DS, and Snyder SH.** Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. *Proc Natl Acad Sci USA* 88: 6368–6371, 1991.
44. **De Giorgi F, Lartigue L, Bauer MK, Schubert A, Grimm S, Hanson GT, Remington SJ, Youle RJ, and Ichas F.** The permeability transition pore signals apoptosis by directing Bax translocation and multimerization. *FASEB J* 16: 607–609, 2002.
45. **De Grey AD.** HO_2·: the forgotten radical. *DNA Cell Biol* 21: 251–257, 2002.
46. **Delettre C, Lenaers G, Griffoin JM, Gigarel N, Lorenzo C, Belenguer P, Pelloquin L, Grosgeorge J, Turc-Carel C, Perret E, Astarie-Dequeker C, Lasquellec L, Arnaud B, Ducommun B, Kaplan J, and Hamel CP.** Nuclear gene OPA1, encoding a mitochondrial dynamin-related protein, is mutated in dominant optic atrophy. *Nat Genet* 26: 207–210, 2000.
47. **Dessolin J, Schuler M, Quinart A, De Giorgi F, Ghosez L, and Ichas F.** Selective targeting of synthetic antioxidants to mitochondria: towards a mitochondrial medicine for neurodegenerative diseases? *Eur J Pharmacol* 447: 155–161, 2002.
48. **Digerness SB, Brookes PS, Goldberg SP, Katholi CR, and Holman WL.** Modulation of mitochondrial adenosine triphosphate-sensitive potassium channels and sodium-hydrogen exchange provide additive protection from severe ischemia-reperfusion injury. *J Thorac Cardiovasc Surg* 125: 863–871, 2003.
49. **Droge W.** Free radicals in the physiological control of cell function. *Physiol Rev* 82: 47–95, 2002.
50. **Duchen MR.** Mitochondria and calcium: from cell signaling to cell death. *J Physiol* 529: 57–68, 2000.
51. **Echtay KS, Roussel D, St-Pierre J, Jekabsons MB, Cadenas S, Stuart JA, Harper JA, Roebuck SJ, Morrison A, Pickering S, Clapham JC, and Brand MD.** Superoxide activates mitochondrial uncoupling proteins. *Nature* 15: 96–99, 2002.
52. **Fantin VR, Berardi MJ, Scorrano L, Korsmeyer SJ, and Leder P.** A novel mitochondriotoxic small molecule that selectively inhibits tumor cell growth. *Cancer Cell* 2: 29–42, 2002.
53. **Favero TG, Zable AC, and Abramson JJ.** Hydrogen peroxide stimulates the Ca^{2+} release channel from skeletal muscle sarcoplasmic reticulum. *J Biol Chem* 270: 25557–25563, 1995.
54. **Fernandez E, Cuenca N, Garcia M, and De Juan J.** Two types of mitochondria are evidenced by protein kinase C immunoreactivity in the Müller cells of the carp retina. *Neurosci Lett* 183: 202–205, 1995.
55. **Fleury C, Mignotte B, and Vayssiere JL.** Mitochondrial reactive oxygen species in cell death signaling. *Biochimie* 84: 131–141, 2002.
56. **Foyouzi-Youssefi R, Arnaudeau S, Borner C, Kelley WL, Tschopp J, Lew DP, Demaurex N, and Krause KH.** Bcl-2 decreases the free Ca^{2+} concentration within the endoplasmic reticulum. *Proc Natl Acad Sci USA* 97: 5723–5728, 2000.
57. **Frank S, Gaume B, Bergmann-Leitner ES, Leitner WW, Robert EG, Catez F, Smith CL, and Youle RJ.** The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis. *Dev Cell* 1: 515–525, 2001.
58. **Ganote CE and Armstrong SC.** Effects of CCCP-induced mitochondrial uncoupling and cyclosporin A on cell volume, cell injury and preconditioning protection of isolated rabbit cardiomyocytes. *J Mol Cell Cardiol* 35: 749–759, 2003.
59. **Ghatan S, Larner S, Kinoshita Y, Hetman M, Patel L, Xia Z, Youle RJ, and Morrison RS.** p38 MAP kinase mediates bax translocation in nitric oxide-induced apoptosis in neurons. *J Cell Biol* 150: 335–347, 2000.
60. **Gincel D, Zaid H, and Shoshan-Barmatz V.** Calcium binding and translocation by the voltage-dependent anion channel: a possible regulatory mechanism in mitochondrial function. *Biochem J* 358: 147–155, 2001.
61. **Green DR and Reed JC.** Mitochondria and apoptosis. *Science* 281: 1309–1311, 1998.
62. **Grijalba MT, Vercesi AE, and Schreier S.** Ca^{2+}-induced increased lipid packing and domain formation in submitochondrial particles. A possible early step in the mechanism of Ca^{2+}-stimulated generation of reactive oxygen species by the respiratory chain. *Biochemistry* 38: 13279–13287, 1999.
63. **Guidot DM, Repine JE, Kitlowski AD, Flores SC, Nelson SK, Wright RM, and McCord JM.** Mitochondrial respiration scavenges extramitochondrial superoxide anion via a nonenzymatic mechanism. *J Clin Invest* 96: 1131–1136, 1995.
64. **Gunter TE, Gunter KK, Sheu SS, and Gavin CE.** Mitochondrial calcium transport: physiological and pathological relevance. *Am J Physiol Cell Physiol* 267: C313–C339, 1994.
65. **Gunter TE, Buntinas L, Sparagna G, Eliseev R, and Gunter K.** Mitochondrial calcium transport: mechanisms and functions. *Cell Calcium* 28: 285–296, 2000.
66. **Gunter TE, Yule DI, Gunter KK, Eliseev RI, and Salter JD.** Calcium and mitochondria. *FEBS Lett* 567: 96–102, 2004.
67. **Hales KG and Fuller MT.** Developmentally regulated mitochondrial fusion mediated by a conserved, novel, predicted GTPase. *Cell* 90: 121–129, 1997.

68. **Halestrap AP.** The mitochondrial permeability transition: its molecular mechanism and role in reperfusion injury. *Biochem Soc Symp* 66: 181–203, 1999.
69. **Halestrap AP and Brennerb C.** The adenine nucleotide translocase: a central component of the mitochondrial permeability transition pore and key player in cell death. *Curr Med Chem* 10: 1507–1525, 2003.
70. **Halliwell B and Gutteridge JMC.** *Free Radicals in Biology and Medicine* (3rd ed.). Oxford: Oxford Univ. Press, 1999.
71. **Han D, Antunes F, Canali R, Rettori D, and Cadenas E.** Voltage-dependent anion channels control the release of the superoxide anion from mitochondria to cytosol. *J Biol Chem* 278: 5557–5563, 2003.
72. **Hansford RG and Zorov D.** Role of mitochondrial calcium transport in the control of substrate oxidation. *Mol Cell Biochem* 184: 359–369, 1998.
73. **Hardy DL, Clark JB, Darley-Usmar VM, and Smith DR.** Reoxygenation of the hypoxic myocardium causes a mitochondrial complex I defect. *Biochem Soc Trans* 18: 549, 1990.
74. **Hardy L, Clark JB, Darley-Usmar VM, Smith DR, and Stone D.** Reoxygenation-dependent decrease in mitochondrial NADH:CoQ reductase (Complex I) activity in the hypoxic/reoxygenated rat heart. *Biochem J* 274: 133–137, 1991.
75. **Harman D.** The free radical theory of aging. *Antioxid Redox Signal* 5: 557–561, 2003.
76. **Harris EJ, Booth R, and Cooper MB.** The effect of superoxide generation on the ability of mitochondria to take up and retain Ca^{2+}. *FEBS Lett* 146: 267–272, 1982.
77. **He H, Li HL, Lin A, and Gottlieb RA.** Activation of the JNK pathway is important for cardiomyocyte death in response to simulated ischemia. *Cell Death Differ* 6: 987–991, 1999.
78. **Hongpaisan J, Winters CA, and Andrews SB.** Calcium-dependent mitochondrial superoxide modulates nuclear CREB phosphorylation in hippocampal neurons. *Mol Cell Neurosci* 24: 1103–1115, 2003.
79. **Huang TT, Yasunami M, Carlson EJ, Gillespie AM, Reaume AG, Hoffman EK, Chan PH, Scott RW, and Epstein CJ.** Superoxide-mediated cytotoxicity in superoxide dismutase-deficient fetal fibroblasts. *Arch Biochem Biophys* 344: 424–432, 1997.
80. **Humphrey SM, Holliss DG, and Cartner LA.** The influence of inhibitors of the ATP degradative pathway on recovery of function and high energy phosphate after transient ischemia in the rat heart. *J Mol Cell Cardiol* 18, *Suppl* 4: 55–59, 1986.
81. **Hunter DR and Haworth RA.** The Ca^{2+}-induced membrane transition in mitochondria. I. The protective mechanisms. *Arch Biochem Biophys* 195: 453–459, 1979.
82. **Hunter DR and Haworth RA.** The Ca^{2+}-induced membrane transition in mitochondria. II. Nature of the Ca^{2+} trigger site. *Arch Biochem Biophys* 195: 460–467, 1979.
83. **Hunter DR and Haworth RA.** The Ca^{2+}-induced membrane transition in mitochondria. III. Transitional Ca^{2+} release. *Arch Biochem Biophys* 195: 468–477, 1979.
84. **Huser J and Blatter LA.** Fluctuations in mitochondrial membrane potential caused by repetitive gating of the permeability transition pore. *Biochem J* 343: 311–317, 1999.
85. **Hwa JJ, Hiller MA, Fuller MT, and Santel A.** Differential expression of the *Drosophila* mitofusin genes *fuzzy onions* (*fzo*) and *dmfn*. *Mech Dev* 116: 213–216, 2002.
86. **Igbavboa U and Pfeiffer DR.** Regulation of reverse uniport activity in mitochondria by extramitochondrial divalent cations. Dependence on a soluble intermembrane space component. *J Biol Chem* 266: 4283–4287, 1991.
87. **Inoue M, Nishikawa M, Sato EF, Ah-Mee P, Kashiba M, Takehara Y, and Utsumi K.** Cross-talk of NO, superoxide and molecular oxygen, a majesty of aerobic life. *Free Radic Res* 31: 251–260, 1999.
88. **Inoue M, Sato EF, Nishikawa M, Park AM, Kira Y, Imada I, and Utsumi K.** Mitochondrial generation of reactive oxygen species and its role in aerobic life. *Curr Med Chem* 10: 2495–505, 2003.
89. **Iverson SL and Orrenius S.** The cardiolipin-cytochrome *c* interaction and the mitochondrial regulation of apoptosis. *Arch Biochem Biophys* 423: 37–46, 2004.
90. **James DI, Parone PA, Mattenberger Y, and Martinou JC.** hFis1, a novel component of the mammalian mitochondrial fission machinery. *J Biol Chem* 278: 36373–36379, 2003.
91. **Jekabsone A, Ivanoviene L, Brown GC, and Borutaite V.** Nitric oxide and calcium together inactivate mitochondrial complex I and induce cytochrome *c* release. *J Mol Cell Cardiol* 35: 803–809, 2003.
92. **Johnston JD and Brand MD.** The mechanism of Ca^{2+} stimulation of citrulline and *N*-acetylglutamate synthesis by mitochondria. *Biochim Biophys Acta* 1033: 85–90, 1990.
93. **Kaminishi T and Kako KJ.** Sensitivity to oxidants of mitochondrial and sarcoplasmic reticular calcium uptake in saponin-treated cardiac myocytes. *Basic Res Cardiol* 84: 282–290, 1989.
94. **Karbowski M, Lee YJ, Gaume B, Jeong SY, Frank S, Nechushtan A, Santel A, Fuller M, Smith CL, and Youle RJ.** Spatial and temporal association of Bax with mitochondrial fission sites, Drp1, and Mfn2 during apoptosis. *J Cell Biol* 159: 931–938, 2002.
95. **Karbowski M and Youle RJ.** Dynamics of mitochondrial morphology in healthy cells and during apoptosis. *Cell Death Differ* 10: 870–880, 2003.
96. **Karmazyn M.** The role of the myocardial sodium-hydrogen exchanger in mediating ischemic and reperfusion injury. From amiloride to cariporide. *Ann NY Acad Sci* 874: 326–334, 1999.
97. **Kelso GF, Porteous CM, Coulter CV, Hughes G, Porteous WK, Ledgerwood EC, Smith RAJ, and Murphy MP.** Selective targeting of a redox-active ubiquinone to mitochondria within cells: antioxidant and antiapoptotic properties. *J Biol Chem* 276: 4588–4596, 2001.
98. **Kim KH, Rodriguez AM, Carrico PM, and Melendez JA.** Potential mechanisms for the inhibition of tumor cell growth by manganese superoxide dismutase. *Antioxid Redox Signal* 3: 361–373, 2001.
99. **Kirichok Y, Krapivinsky G, and Clapham DE.** The mitochondrial calcium uniporter is a highly selective ion channel. *Nature* 427: 360–364, 2004.
100. **Knyazeva TA, Malyugin EF, Zarinskaya SA, and Archakov AI.** Solubilization of cytochrome *c* in ischemic liver tissue. *Vopr Med Khim* 21: 481–485, 1975.
101. **Kokoszka JE, Waymire KG, Levy SE, Sligh JE, Cai J, Jones DP, MacGregor GR, and Wallace DC.** The ADP/ATP translocator is not essential for the mitochondrial permeability transition pore. *Nature* 427: 461–465, 2004.
102. **Kowaltowski AJ, Naia-da-Silva ES, Castilho RF, and Vercesi AE.** Ca^{2+}-stimulated mitochondrial reactive oxygen species generation and permeability transition are inhibited by dibucaine or Mg^{2+}. *Arch Biochem Biophys* 359: 77–81, 1998.
103. **Kulisz A, Chen N, Chandel NS, Shao Z, and Schumacker PT.** Mitochondrial ROS initiate phosphorylation of p38 MAP kinase during hypoxia in cardiomyocytes. *Am J Physiol Lung Cell Mol Physiol* 282: L1324–L1329, 2002.
104. **Legros F, Lombes A, Frachon P, and Rojo M.** Mitochondrial fusion in human cells is efficient, requires the inner membrane potential, and is mediated by mitofusins. *Mol Biol Cell* 13: 4343–4354, 2002.
105. **Lesnefsky EJ, Moghaddas S, Tandler B, Kerner J, and Hoppel CL.** Mitochondrial dysfunction in cardiac disease: ischemia-reperfusion, aging, and heart failure. *J Mol Cell Cardiol* 33: 1065–1089, 2001.
106. **Levi S, Corsi B, Bosisio M, Invernizzi R, Volz A, Sanford D, Arosio P, and Drysdale J.** A human mitochondrial ferritin encoded by an intronless gene. *J Biol Chem* 276: 24437–24440, 2001.
107. **Levonen AL, Patel RP, Brookes P, Go YM, Jo H, Parthasarathy S, Anderson PG, and Darley-Usmar VM.** Mechanisms of cell signaling by nitric oxide and peroxynitrite: from mitochondria to MAP kinases. *Antioxid Redox Signal* 3: 215–229, 2001.
108. **Li N, Ragheb K, Lawler G, Sturgis J, Rajwa B, Melendez JA, and Robinson JP.** Mitochondrial complex I inhibitor rotenone induces apoptosis through enhancing mitochondrial reactive oxygen species production. *J Biol Chem* 278: 8516–8525, 2003.
109. **Lin TK, Hughes G, Muratovska A, Blaikie FH, Brookes PS, Darley-Usmar V, Smith RA, and Murphy MP.** Specific modification of mitochondrial protein thiols in response to oxidative stress: a proteomics approach. *J Biol Chem* 277: 17048–17056, 2002.
110. **Liu X, Kim CN, Yang J, Jemmerson R, and Wang X.** Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome *c*. *Cell* 86: 147–157, 1996.
111. **Lizard G, Fournel S, Genestier L, Dhedin N, Chaput C, Flacher M, Mutin M, Panaye G, and Revillard JP.** Kinetics of plasma membrane and mitochondrial alterations in cells undergoing apoptosis. *Cytometry* 21: 275–283, 1995.
112. **Loeffler M and Kroemer G.** The mitochondrion in cell death control: certainties and incognita. *Exp Cell Res* 256: 19–26, 2000.
113. **Maechler P.** Mitochondria as the conductor of metabolic signals for insulin exocytosis in pancreatic beta-cells. *Cell Mol Life Sci* 59: 1803–1818, 2002.

114. **Mailer K.** Superoxide radical as electron donor for oxidative phosphorylation of ADP. *Biochem Biophys Res Commun* 170: 59–64, 1990.
115. **Mannella CA, Buttle K, Rath BK, and Marko M.** Electron microscopic tomography of rat-liver mitochondria and their interaction with the endoplasmic reticulum. *Biofactors* 8: 225–228, 1998.
116. **Martinou JC, Desagher S, and Antonsson B.** Cytochrome *c* release from mitochondria: all or nothing. *Nat Cell Biol* 2: E41–E43, 2000.
117. **Mattiasson G, Shamloo M, Gido G, Mathi K, Tomasevic G, Yi S, Warden CH, Castilho RF, Melcher T, Gonzalez-Zulueta M, Nikolich K, and Wieloch T.** Uncoupling protein-2 prevents neuronal death and diminishes brain dysfunction after stroke and brain trauma. *Nat Med* 9: 1062–1068, 2003.
118. **McCormack JG and Denton RM.** Mitochondrial Ca^{2+} transport and the role of intramitochondrial Ca^{2+} in the regulation of energy metabolism. *Dev Neurosci* 15: 165–173, 1993.
119. **McGivan JD, Bradford NM, and Mendes-Mourao J.** The regulation of carbamoyl phosphate synthase activity in rat liver mitochondria. *Biochem J* 154: 415–421, 1976.
120. **McNaught KS and Brown GC.** Nitric oxide causes glutamate release from brain synaptosomes. *J Neurochem* 70: 1541–1546, 1998.
121. **Melov S, Coskun P, Patel M, Tuinstra R, Cottrell B, Jun AS, Zastawny TH, Dizdaroglu M, Goodman SI, Huang TT, Miziorko H, Epstein CJ, and Wallace DC.** Mitochondrial disease in superoxide dismutase 2 mutant mice. *Proc Natl Acad Sci USA* 96: 846–851, 1999.
122. **Meredith MJ and Reed DJ.** Depletion in vitro of mitochondrial glutathione in rat hepatocytes and enhancement of lipid peroxidation by adriamycin and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). *Biochem Pharmacol* 32: 1383–1388, 1982.
123. **Mildaziene V, Baniene R, Nauciene Z, Bakker BM, Brown GC, Westerhoff HV, and Kholodenko BN.** Calcium indirectly increases the control exerted by the adenine nucleotide translocator over 2-oxoglutarate oxidation in rat heart mitochondria. *Arch Biochem Biophys* 324: 130–134, 1995.
124. **Mironova GD, Baumann M, Kolomytkin O, Krasichkova Z, Berdimuratov A, Sirota T, Virtanen I, and Saris NE.** Purification of the channel component of the mitochondrial calcium uniporter and its reconstitution into planar lipid bilayers. *J Bioenerg Biomembr* 26: 231–238, 1994.
125. **Miwa S and Brand MD.** Mitochondrial matrix reactive oxygen species production is very sensitive to mild uncoupling. *Biochem Soc Trans* 31: 1300–1301, 2003.
126. **Montero M, Lobaton CD, Moreno A, and Alvarez J.** A novel regulatory mechanism of the mitochondrial Ca^{2+} uniporter revealed by the p38 mitogen-activated protein kinase inhibitor SB202190. *FASEB J* 16: 1955–1957, 2002.
127. **Moore CL.** Specific inhibition of mitochondrial Ca^{++} transport by ruthenium red. *Biochem Biophys Res Commun* 42: 298–305, 1971.
128. **Muller FL, Roberts AG, Bowman MK, and Kramer DM.** Architecture of the Q_o site of the cytochrome bc_1 complex probed by superoxide production. *Biochemistry* 42: 6493–6499, 2003.
129. **Murphy E.** Primary and secondary signaling pathways in early preconditioning that converge on the mitochondria to produce cardioprotection. *Circ Res* 94: 7–16, 2004.
130. **Murphy MP, Echtay KS, Blaikie FH, Asin-Cayuela J, Cocheme HM, Green K, Buckingham JA, Taylor ER, Hurrell F, Hughes G, Miwa S, Cooper CE, Svistunenko DA, Smith RA, and Brand MD.** Superoxide activates uncoupling proteins by generating carbon-centered radicals and initiating lipid peroxidation: studies using a mitochondria-targeted spin trap derived from α-phenyl-*N*-*tert*-butylnitrone. *J Biol Chem* 278: 48534–48545, 2003.
131. **Naito Z, Kudo M, Xu G, Nishigaki R, Yokoyama M, Yamada N, and Asano G.** Immunohistochemical localization of mitogen-activated protein kinase (MAPK) family and morphological changes in rat heart after ischemia-reperfusion injury. *Med Electron Microsc* 33: 74–81, 2000.
132. **Narita M, Shimizu S, Ito T, Chittenden T, Lutz RJ, Matsuda H, and Tsujimoto Y.** Bax interacts with the permeability transition pore to induce permeability transition and cytochrome *c* release in isolated mitochondria. *Proc Natl Acad Sci USA* 95: 14681–14686, 1998.
133. **Negre-Salvayre A, Hirtz C, Carrera G, Cazenave R, Troly M, Salvayre R, Penicaud L, and Casteilla L.** A role for uncoupling protein-2 as a regulator of mitochondrial hydrogen peroxide generation. *FASEB J* 11: 809–815, 1997.
134. **Nicholls DG and Budd SL.** Mitochondria and neuronal survival. *Physiol Rev* 80: 315–360, 2000.
135. **Nicholls DG, Budd SL, Ward MW, and Castilho RF.** Excitotoxicity and mitochondria. *Biochem Soc Symp* 66: 55–67, 1999.
136. **Nicholls DG and Ferguson S.J.** *Bioenergetics 2.* San Diego, CA: Academic, 1992.
137. **Nicotera P, Leist M, and Ferrando-May E.** Intracellular ATP, a switch in the decision between apoptosis and necrosis. *Toxicol Lett* 102–103: 139–142, 1998.
138. **Nunnari J, Marshall WF, Straight A, Murray A, Sedat JW, and Walter P.** Mitochondrial transmission during mating in *Saccharomyces cerevisiae* is determined by mitochondrial fusion and fission and the intramitochondrial segregation of mitochondrial DNA. *Mol Biol Cell* 8: 1233–1242, 1997.
139. **Okado-Matsumoto A and Fridovich I.** Subcellular distribution of superoxide dismutases (SOD) in rat liver: Cu,Zn-SOD in mitochondria. *J Biol Chem* 276: 38388–38393, 2001.
140. **Okuda M, Lee HC, Kumar C, and Chance B.** Comparison of the effect of a mitochondrial uncoupler, 2,4-dinitrophenol and adrenaline on oxygen radical production in the isolated perfused rat liver. *Acta Physiol Scand* 145: 159–168, 1992.
141. **O'Reilly CM, Fogarty KE, Drummond RM, Tuft RA, and Walsh JV Jr.** Quantitative analysis of spontaneous mitochondrial depolarizations. *Biophys J* 85: 3350–3357, 2003.
142. **Otsuga D, Keegan BR, Brisch E, Thatcher JW, Hermann GJ, Bleazard W, and Shaw JM.** The dynamin-related GTPase, Dnm1p, controls mitochondrial morphology in yeast. *J Cell Biol* 143: 333–349, 1998.
143. **Ott M, Robertson JD, Gogvadze V, Zhivotovsky B, and Orrenius S.** Cytochrome *c* release from mitochondria proceeds by a two-step process. *Proc Natl Acad Sci USA* 99: 1259–1263, 2002.
144. **Packer MA and Murphy MP.** Peroxynitrite causes calcium efflux from mitochondria which is prevented by cyclosporin A. *FEBS Lett* 345: 237–240, 1994.
145. **Papa S, Scacco S, Sardanelli AM, Petruzzella V, Vergari R, Signorile A, and Technikova-Dobrova Z.** Complex I and the cAMP cascade in human physiopathology. *Biosci Rep* 22: 3–16, 2002.
146. **Patterson SD, Spahr CS, Daugas E, Susin SA, Irinopoulou T, Koehler C, and Kroemer G.** Mass spectrometric identification of proteins released from mitochondria undergoing permeability transition. *Cell Death Differ* 7: 137–144, 2000.
147. **Pereverzev MO, Vygodina TV, Konstantinov AA, and Skulachev VP.** Cytochrome *c*, an ideal antioxidant. *Biochem Soc Trans* 31: 1312–1315, 2003.
148. **Perez-Campo R, Lopez-Torres M, Cadenas S, Rojas C, and Barja G.** The rate of free radical production as a determinant of the rate of aging: evidence from the comparative approach. *J Comp Physiol [B]* 168: 149–158, 1998.
149. **Pieper AA, Verma A, Zhang J, and Snyder SH.** Poly (ADP-ribose) polymerase, nitric oxide and cell death. *Trends Pharmacol Sci* 20: 171–181, 1999.
150. **Pinton P, Ferrari D, Magalhaes P, Schulze-Osthoff K, Di Virgilio F, Pozzan T, and Rizzuto R.** Reduced loading of intracellular Ca^{2+} stores and downregulation of capacitative Ca^{2+} influx in Bcl-2-overexpressing cells. *J Cell Biol* 148: 857–862, 2000.
151. **Pitts KR, Yoon Y, Krueger EW, and McNiven MA.** The dynamin-like protein DLP1 is essential for normal distribution and morphology of the endoplasmic reticulum and mitochondria in mammalian cells. *Mol Biol Cell* 10: 4403–4417, 1999.
152. **Pomytkin IA and Kolesova OE.** Key role of succinate dehydrogenase in insulin-induced inactivation of protein tyrosine phosphatases. *Bull Exp Biol Med* 133: 568–570, 2002.
153. **Raha S, Myint AT, Johnstone L, and Robinson BH.** Control of oxygen free radical formation from mitochondrial complex I: roles for protein kinase A and pyruvate dehydrogenase kinase. *Free Radic Biol Med* 32: 421–430, 2002.
154. **Ramachandran A, Moellering D, Go YM, Shiva S, Levonen AL, Jo H, Patel RP, Parthasarathy S, and Darley-Usmar VM.** Activation of c-Jun N-terminal kinase and apoptosis in endothelial cells mediated by endogenous generation of hydrogen peroxide. *Biol Chem* 383: 693–701, 2002.
155. **Ranganathan AC, Nelson KK, Rodriguez AM, Kim KH, Tower GB, Rutter JL, Brinckerhoff CE, Huang TT, Epstein CJ, Jeffrey JJ, and Melendez JA.** Manganese superoxide dismutase signals matrix metalloproteinase expression via H_2O_2-dependent ERK1/2 activation. *J Biol Chem* 276: 14264–14270, 2001.

156. **Reed KC and Bygrave FLA.** A low molecular weight ruthenium complex inhibitory to mitochondrial Ca^{2+} transport. *FEBS Lett* 46: 109–114, 1974.
157. **Richter C.** Reactive oxygen and nitrogen species regulate mitochondrial Ca^{2+} homeostasis and respiration. *Biosci Rep* 17: 53–66, 1997.
158. **Rojo M, Legros F, Chateau D, and Lombes A.** Membrane topology and mitochondrial targeting of mitofusins, ubiquitous mammalian homologs of the transmembrane GTPase Fzo. *J Cell Sci* 115: 1663–1674, 2002.
159. **Rothstein EC, Byron KL, Reed RE, Fliegel L, and Lucchesi PA.** H_2O_2-induced Ca^{2+} overload in NRVM involves ERK1/2 MAP kinases: role for an NHE-1-dependent pathway. *Am J Physiol Heart Circ Physiol* 283: H598–H605, 2002.
160. **Ruben L, Goodman DB, and Rasmussen H.** Identification of calmodulin in mitochondria from rat liver: a possible role in regulation of the oligomycin sensitive ATPase. *Ann NY Acad Sci* 356: 427–428, 1980.
161. **Sadek HA, Nulton-Persson AC, Szweda PA, and Szweda LI.** Cardiac ischemia/reperfusion, aging, and redox-dependent alterations in mitochondrial function. *Arch Biochem Biophys* 420: 201–208, 2003.
162. **Saksela M, Lapatto R, and Raivio KO.** Irreversible conversion of xanthine dehydrogenase into xanthine oxidase by a mitochondrial protease. *FEBS Lett* 443: 117–120, 1999.
163. **Santel A, Frank S, Gaume B, Herrler M, Youle RJ, and Fuller MT.** Mitofusin-1 protein is a generally expressed mediator of mitochondrial fusion in mammalian cells. *J Cell Sci* 116: 2763–2774, 2003.
164. **Santel A and Fuller MT.** Control of mitochondrial morphology by a human mitofusin. *J Cell Sci* 114: 867–874, 2001.
165. **Saris NE, Sirota TV, Virtanen I, Niva K, Penttila T, Dolgachova LP, and Mironova GD.** Inhibition of the mitochondrial calcium uniporter by antibodies against a 40-kDa glycoprotein. *J Bioenerg Biomembr* 25: 307–312, 1993.
166. **Sattler R and Tymianski M.** Molecular mechanisms of glutamate receptor-mediated excitotoxic neuronal cell death. *Mol Neurobiol* 24: 107–129, 2001.
167. **Sauer H, Wartenberg M, and Hescheler J.** Reactive oxygen species as intracellular messengers during cell growth and differentiation. *Cell Physiol Biochem* 11: 173–186, 2001.
168. **Savabi F and Bessman SP.** Recovery of isolated rat atrial function related to ATP under different anoxic conditions. *Arch Biochem Biophys* 248: 151–157, 1986.
169. **Scarpa A and Azzone GF.** The mechanism of ion translocation in mitochondria. 4. Coupling of K^+ efflux with Ca^{2+} uptake. *Eur J Biochem* 12: 328–335, 1970.
170. **Scheffler IE.** *Mitochondria* (1st ed.). New York: Wiley, 1999.
171. **Schwerzmann K, Cruz-Orive LM, Eggman R, Sanger A, and Weibel ER.** Molecular architecture of the inner membrane of mitochondria from rat liver: a combined biochemical and stereological study. *J Cell Biol* 102: 97–103, 1986.
172. **Scorrano L, Oakes SA, Opferman JT, Cheng EH, Sorcinelli MD, Pozzan T, and Korsmeyer SJ.** BAX and BAK regulation of endoplasmic reticulum Ca^{2+}: a control point for apoptosis. *Science* 300: 135–139, 2003.
173. **Sesaki H and Jensen RE.** Division versus fusion: Dnm1p and Fzo1p antagonistically regulate mitochondrial shape. *J Cell Biol* 147: 699–706, 1999.
174. **Sesaki H, Southard SM, Yaffe MP, and Jensen RE.** Mgm1p, a dynamin-related GTPase, is essential for fusion of the mitochondrial outer membrane. *Mol Biol Cell* 14: 2342–2356, 2003.
175. **Shan X, Jones DP, Hashmi M, and Anders MW.** Selective depletion of mitochondrial glutathione concentrations by (*R,S*)-3-hydroxy-4-pentenoate potentiates oxidative cell death. *Chem Res Toxicol* 6: 75–81, 1993.
176. **Shi YF, Sahai BM, and Green DR.** Cyclosporin A inhibits activation-induced cell death in T-cell hybridomas and thymocytes. *Nature* 339: 625–626, 1989.
177. **Shimizu S, Narita M, and Tsujimoto Y.** Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. *Nature* 399: 483–487, 1999.
178. **Shiva S and Darley-Usmar VM.** Control of the nitric oxide-cytochrome *c* oxidase signaling pathway under pathological and physiological conditions. *IUBMB Life* 55: 585–590, 2003.
179. **Smirnova E, Griparic L, Shurland DL, and van der Bliek AM.** Dynamin-related protein drp1 is required for mitochondrial division in mammalian cells. *Mol Biol Cell* 12: 2245–2256, 2001.
180. **Smirnova E, Shurland DL, Ryazantsev SN, and van der Bliek AM.** A human dynamin-related protein controls the distribution of mitochondria. *J Cell Biol* 143: 351–358, 1998.
181. **Smith RA, Porteous CM, Coulter CV, and Murphy MP.** Selective targeting of an antioxidant to mitochondria. *Eur J Biochem* 263: 709–716, 1999.
182. **Smith RA, Porteous CM, Gane AM, and Murphy MP.** Delivery of bioactive molecules to mitochondria in vivo. *Proc Natl Acad Sci USA* 100: 5407–5412, 2003.
183. **Snabaitis AK, Hearse DJ, and Avkiran M.** Regulation of sarcolemmal Na^+/H^+ exchange by hydrogen peroxide in adult rat ventricular myocytes. *Cardiovasc Res* 53: 470–480, 2002.
184. **Sohal RS and Allen RG.** Relationship between metabolic rate, free radicals, differentiation and aging: a unified theory. *Basic Life Sci* 35: 75–104, 1995.
185. **Sousa SC, Maciel EN, Vercesi AE, and Castilho RF.** Ca^{2+}-induced oxidative stress in brain mitochondria treated with the respiratory chain inhibitor rotenone. *FEBS Lett* 543: 179–183, 2003.
186. **Sparagna GC, Gunter KK, Sheu SS, and Gunter TE.** Mitochondrial calcium uptake from physiological-type pulses of calcium. A description of the rapid uptake mode. *J Biol Chem* 270: 27510–27515, 1995.
187. **Starkov AA and Fiskum G.** Regulation of brain mitochondrial H_2O_2 production by membrane potential and NAD(P)H redox state. *J Neurochem* 86: 1101–1107, 2003.
188. **Starkov AA, Polster BM, and Fiskum G.** Regulation of hydrogen peroxide production by brain mitochondria by calcium and Bax. *J Neurochem* 83: 220–228, 2002.
189. **Stone D, Darley-Usmar V, Smith DR, and O'Leary V.** Hypoxia-reoxygenation induced increase in cellular Ca^{2+} in myocytes and perfused hearts: the role of mitochondria. *J Mol Cell Cardiol* 21: 963–973, 1989.
190. **Stone D, Darley-Usmar VM, and Martin JF.** Calcium fluxes and reperfusion damage: the role of mitochondria. In: *Myocardial Response to Acute Injury.* edited by Parratt JR. Basingstoke, UK: Macmillan, 1992, p. 67–82.
191. **Stout AK, Raphael HM, Kanterewicz BI, Klann E, and Reynolds IJ.** Glutamate-induced neuron death requires mitochondrial calcium uptake. *Nat Neurosci* 1: 366–373, 1998.
192. **St-Pierre J, Buckingham JA, Roebuck SJ, and Brand MD.** Topology of superoxide production from different sites in the mitochondrial electron transport chain. *J Biol Chem* 277: 44784–44790, 2002.
193. **Sun J, Xu L, Eu JP, Stamler JS, and Meissner G.** Classes of thiols that influence the activity of the skeletal muscle calcium release channel. *J Biol Chem* 276: 15625–15630, 2001.
194. **Szabo C and Dawson VL.** Role of poly(ADP-ribose) synthetase in inflammation and ischaemia-reperfusion. *Trends Pharmacol Sci* 19: 287–298, 1998.
195. **Szewczyk A and Marban E.** Mitochondria: a new target for K channel openers? *Trends Pharmacol Sci* 20: 157–161, 1999.
196. **Szewczyk A and Wojtczak L.** Mitochondria as a pharmacological target. *Pharmacol Rev* 54: 101–27, 2002.
197. **Taylor ER, Hurrell F, Shannon RJ, Lin TK, Hirst J, and Murphy MP.** Reversible glutathionylation of complex I increases mitochondrial superoxide formation. *J Biol Chem* 278: 19603–19610, 2003.
198. **Territo PR, French SA, Dunleavy MC, Evans FJ, and Balaban RS.** Calcium activation of heart mitochondrial oxidative phosphorylation: rapid kinetics of $m\dot{V}O_2$, NADH, and light scattering. *J Biol Chem* 276: 2586–2599, 2001.
199. **Teshima Y, Akao M, Jones SP, and Marban E.** Uncoupling protein-2 overexpression inhibits mitochondrial death pathway in cardiomyocytes. *Circ Res* 93: 192–200, 2003.
200. **Tsujimoto Y and Shimizu S.** Bcl-2 family: life-or-death switch. *FEBS Lett* 466: 6–10, 2000.
201. **Turrens JF.** Superoxide production by the mitochondrial respiratory chain. *Biosci Rep* 17: 3–8, 1997.
202. **Turrens JF.** Mitochondrial formation of reactive oxygen species. *J Physiol* 552: 335–344, 2003.
203. **Ueda S, Masutani H, Nakamura H, Tanaka T, Ueno M, and Yodoi J.** Redox control of cell death. *Antioxid Redox Signal* 4: 405–414, 2002.
204. **Vercesi AE, Kowaltowski AJ, Grijalba MT, Meinicke AR, and Castilho RF.** The role of reactive oxygen species in mitochondrial permeability transition. *Biosci Rep* 17: 43–52, 1997.
205. **Vergun O, Votyakova TV, and Reynolds IJ.** Spontaneous changes in mitochondrial membrane potential in single isolated brain mitochondria. *Biophys J* 85: 3358–3366, 2003.

206. **Vinogradov A and Scarpa A.** The initial velocities of calcium uptake by rat liver mitochondria. *J Biol Chem* 248: 5527–5531, 1973.
207. **Virag L, Salzman AL, and Szabo C.** Poly(ADP-ribose) synthetase activation mediates mitochondrial injury during oxidant-induced cell death. *J Immunol* 161: 3753–3759, 1998.
208. **Waterhouse NJ, Goldstein JC, von Ahsen O, Schuler M, Newmeyer DD, and Green DR.** Cytochrome *c* maintains mitochondrial transmembrane potential and ATP generation after outer mitochondrial membrane permeabilization during the apoptotic process. *J Cell Biol* 153: 319–328, 2001.
209. **Weissig V.** Mitochondrial-targeted drug and DNA delivery. *Crit Rev Ther Drug Carrier Syst* 20: 1–62, 2003.
210. **Weissig V, Boddapati SV, D'Souza GGM, and Cheng SM.** Targeting of low-molecular weight drugs to mammalian mitochondria. *Drug Design Rev Online* 1: 15–28, 2004.
211. **Wernette ME, Ochs RS, and Lardy HA.** Ca^{2+} stimulation of rat liver mitochondrial glycerophosphate dehydrogenase. *J Biol Chem* 256: 12767–12771, 1981.
212. **Wikstrom M and Saari H.** A spectral shift in cytochrome *a* induced by calcium ions. *Biochim Biophys Acta* 408: 170–179, 1975.
213. **Wong ED, Wagner JA, Scott SV, Okreglak V, Holewinske TJ, Cassidy-Stone A, and Nunnari J.** The intramitochondrial dynamin-related GTPase, Mgm1p, is a component of a protein complex that mediates mitochondrial fusion. *J Cell Biol* 160: 303–11, 2003.
214. **Xu L, Eu JP, Meissner G, and Stamler JS.** Activation of the cardiac calcium release channel (ryanodine receptor) by poly-S-nitrosylation. *Science* 279: 234–237, 1998.
215. **Yellon DM and Downey JM.** Preconditioning the myocardium: from cellular physiology to clinical cardiology. *Physiol Rev* 83:1113–1151, 2003.
216. **Ying WL, Emerson J, Clarke MJ, and Sanadi DR.** Inhibition of mitochondrial calcium ion transport by an oxo-bridged dinuclear ruthenium ammine complex. *Biochemistry* 30: 4949–4952, 1991.
217. **Yoon Y, Krueger EW, Oswald BJ, and McNiven MA.** The mitochondrial protein hFis1 regulates mitochondrial fission in mammalian cells through an interaction with the dynamin-like protein DLP1. *Mol Cell Biol* 23: 5409–5420, 2003.
218. **Yoon Y and McNiven MA.** Mitochondrial division: new partners in membrane pinching. *Curr Biol* 11: R67–R70, 2001.
219. **Yoon Y, Pitts KR, and McNiven MA.** Mammalian dynamin-like protein DLP1 tubulates membranes. *Mol Biol Cell* 12: 2894–2905, 2001.
220. **Yu SW, Wang H, Poitras MF, Coombs C, Bowers WJ, Federoff HJ, Poirier GG, Dawson TM, and Dawson VL.** Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. *Science* 297: 259–263, 2002.
221. **Yue TL, Wang C, Gu JL, Ma XL, Kumar S, Lee JC, Feuerstein GZ, Thomas H, Maleeff B, and Ohlstein EH.** Inhibition of extracellular signal-regulated kinase enhances ischemia/reoxygenation-induced apoptosis in cultured cardiac myocytes and exaggerates reperfusion injury in isolated perfused heart. *Circ Res* 86: 692–699, 2000.
222. **Zamzami N, Marchetti P, Castedo M, Hirsch T, Susin SA, Masse B, and Kroemer G.** Inhibitors of permeability transition interfere with the disruption of the mitochondrial transmembrane potential during apoptosis. *FEBS Lett* 384: 53–57, 1996.
223. **Zaugg M and Schaub MC.** Signaling and cellular mechanisms in cardiac protection by ischemic and pharmacological preconditioning. *J Muscle Res Cell Motil* 24: 219–249, 2003.
224. **Zoccarato F, Cavallini L, and Alexandre A.** Respiration-dependent removal of exogenous H_2O_2 in brain mitochondria: inhibition by Ca^{2+}. *J Biol Chem* 279: 4166–4174, 2004.