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Forum Review

A Wave of Reactive Oxygen Species (ROS)-Induced ROS Release in a Sea of Excitable Mitochondria

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ABSTRACT

Once considered simply as the main source of ATP, mitochondria are now implicated in the control of many additional aspects of cell physiology, such as calcium signaling, and pathology, as in injury incurred on ischemia and subsequent reperfusion (I/R). Mitochondrial respiration is ordinarily accompanied by low-level ROS production, but they can respond to elevated ROS concentrations by increasing their own ROS production, a phenomenon termed ROS-induced ROS release (RIRR). Two modes of RIRR have been described. In the first mode of RIRR, enhanced ROS leads to mitochondrial depolarization via activation of the MPTP, yielding a short-lived burst of ROS originating from the mitochondrial electron transport chain (ETC). The second mode of RIRR is MPTP independent but is regulated by the mitochondrial benzodiazepine receptor (mBzR). Increased ROS in the mitochondrion triggers opening of the inner mitochondrial membrane anion channel (IMAC), resulting in a brief increase in ETC-derived ROS. Both modes of RIRR have been shown to transmit localized mitochondrial perturbations throughout the cardiac cell in the form of oscillations or waves but are kinetically distinct and may involve different ROS that serve as second messengers. In this review, we discuss the mechanisms of these different modes of RIRR. *Antioxid. Redox Signal.* 8, 1651–1665.

INTRODUCTION

Once considered simply as the main source of ATP, mitochondria are now implicated in the control of many additional aspects of cell physiology, such as calcium signaling, and pathology, as in injury incurred on ischemia and subsequent reperfusion (I/R). During I/R injury, the heart initially experiences a deprivation of coronary blood flow (ischemia). Subsequent rescue of the heart requires the restoration of blood supply (reperfusion). However, reoxygenation results in a dramatic increase in reactive oxygen species (ROS) generation by the mitochondria, which, depending on the severity and duration of ischemia, may lead to either programmed cell death or a preconditioning response that may prevent cell death after a subsequent more severe ischemia.

Although normal mitochondrial respiration is associated with low-level ROS production (23), mitochondria also exhibit ROS excitability [*i.e.*, they can respond to elevated ROS concentrations, either exogenous (93) or endogenous (79), by increasing their own ROS production, a phenomenon termed ROS-induced ROS release (RIRR) (79, 93, 126)].

Two modes of RIRR have been described. The first mode of RIRR is mitochondrial permeability transition pore (MPTP) dependent. Enhanced ROS leads to mitochondrial depolarization via activation of the MPTP, which in turn yields a short-lived burst of ROS originating from the mitochondrial electron transport chain (ETC) (126). MPTP-medi-

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ated RIRR has been linked to both the preconditioning and the apoptotic pathways in cardiac cells (37, 66, 107).

The second mode of RIRR is MPTP independent. Increased ROS presence in the mitochondrion results in the opening of the inner mitochondrial membrane anion channel (IMAC), resulting in a brief increase in ETC-derived ROS. Ligand studies show that this RIRR mode is regulated by the mitochondrial benzodiazepine receptor (mBzR) (6, 34) and participates in I/R-induced myocardial arrhythmias (1). Both modes of RIRR have been shown to transmit localized mitochondrial perturbations throughout the cardiac cell in the form of oscillations or waves (6, 24). However, mitochondrion-to-mitochondrion transmission of MPTP-mediated RIRR is kinetically distinct from IMAC-mediated RIRR and may involve different ROS that serve as second messengers.

In this review, we discuss the mechanisms of these different modes of RIRR. Moreover, we focus on the role of RIRR in protective and cell-death pathways activated by I/R.

MAIN FUNCTION OF THE MITOCHONDRIA

Cardiac mitochondria exhibit a distinctive arrangement and morphology. The adult cardiomyocyte contains approximately 7,000 electrically independent (unpublished results) mitochondria, which are positioned along the myofibrils in a highly ordered manner (Fig. 1a). Cardiac mitochondria are attached to the cytoskeleton (90), to maintain a static arrangement that is highly regular (121), contrasted with neuronal mitochondria, which are filamentous and extremely mobile (59). The precise positioning and abundance of mitochondria may ensure a highly efficient localized ATP delivery in the high-workload cardiomyocyte (3). In addition to their role as free-energy harvesters, mitochondria play a key role in calcium signaling (42), and much of the renewed interest in mitochondria stems from their participation in programmed cell death, or apoptosis (51).

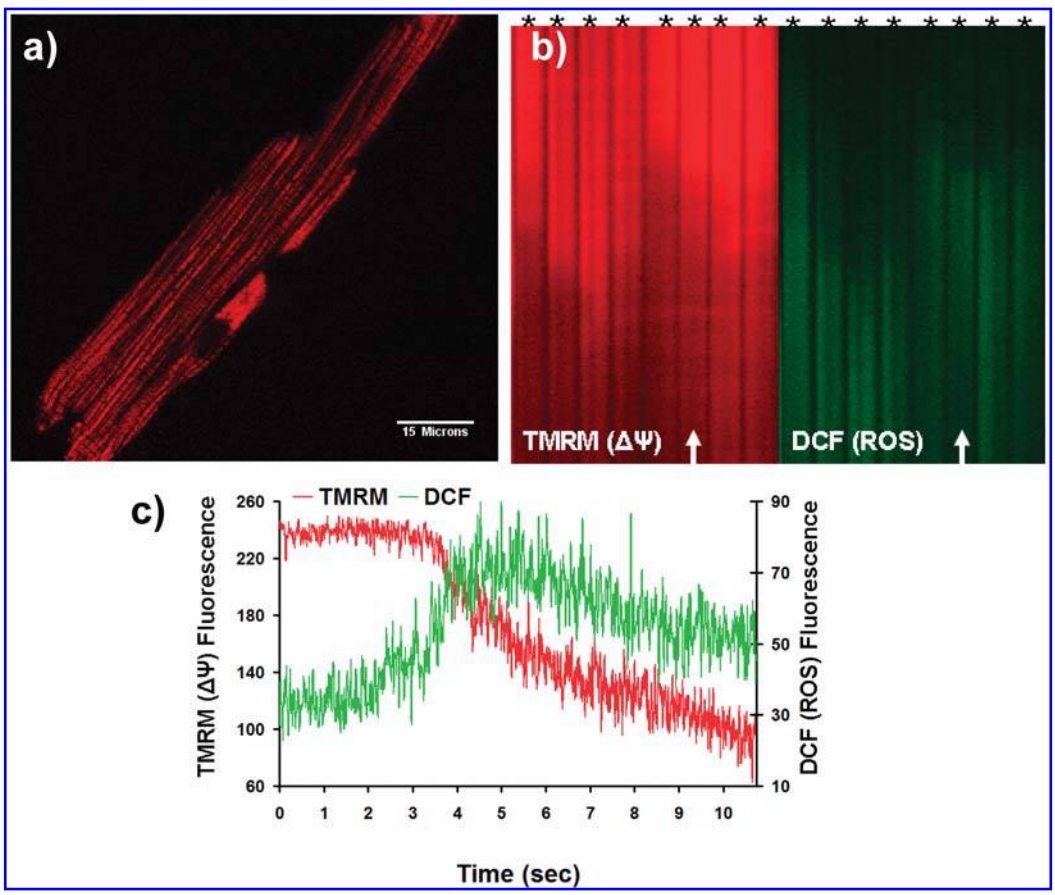

FIG. 1. In situ determination of ROS-induced ROS release in the adult cardiomyocyte. An adult rat cardiomyocyte, preloaded with 100 nM TMRM and 10 μM H_2DCF-DA. **(a)** Organization of the mitochondria of an isolated cardiomyocyte. Confocal imaging of TMRM-labeled mitochondria. Intermyofibrillar mitochondria are arranged in rows, and perinuclear mitochondria are densely packed around the nucleus. **(b)** A single 15-μm row of mitochondria was confocal line-scanned for 10.75 s, exciting both TMRM and DCF. Individual mitochondria are labeled by an asterisk. **(c)** Plot profile of RIRR kinetics at the level of the single mitochondrion **(b,** arrows**).** (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars.)

ATP generation

In the terminally differentiated cardiomyocyte, mitochondria are responsible for almost all the supply of ATP, the free-energy carrier fuelling most cellular energy-dependent operations. To maintain contraction, cardiomyocytes must continually replenish their supply of ATP, which would be exhausted within seconds in the absence of resynthesis.

Under fasting conditions, mitochondria obtain their principal oxidative substrates from glycolysis through pyruvate (40%) and from fatty acids (60–70%) (96). Pyruvate is imported into the mitochondrial matrix, where it undergoes oxidative decarboxylation to acetyl CoA. Fatty acids are imported into the mitochondrial matrix via carnitine palmitoyl transferase enzymes or other transporters, and the acyl CoA thioester is cleaved two carbons at a time to yield successive molecules of acetyl CoA in the beta oxidation spiral. The acetyl CoA enters the tricarboxylic acid (TCA) cycle and is oxidized to carbon dioxide, thereby reducing free NAD^+ and FAD bound to succinate dehydrogenase. Electrons from NADH are transferred to complex I; $FADH_2$ is part of complex II. Electrons from complexes I and II are transferred through ubiquinone/ol (Q pool) to complex III, and cytochrome *c* shuttles electrons from complex III to complex IV. Ultimately, complex IV uses molecular oxygen (O_2) as the terminal electron acceptor, reducing O_2 to water (H_2O).

The free energy that becomes available during electron transport is used by complexes I, III and IV to extrude protons from the matrix to the intermembrane space (IMS), forming the proton motive force ($\Delta\mu H^+$). $\Delta\mu H^+$ describes the electrochemical potential difference for protons across the inner mitochondrial membrane (IMM), which represents the net electric potential difference across the IMM ($\Delta\Psi_m$) and the difference in pH (ΔpH). Both of these are the result of proton extrusion. In cardiomyocyte mitochondria, $\Delta\Psi_m$ has been measured at 150 mV and ΔpH at 0.53 pH units (68). The approximately 18 kJ/mol of free energy contained within $\Delta\mu H^+$ can be used to drive H^+ back through the F_1F_0 ATPase, whereby ADP is phosphorylated to generate ATP. The adenine nucleotide translocator (ANT), which is located in the IMM, then exchanges ATP for ADP, thereby transferring ATP to the IMS, where via coupling with creatine kinase systems, its free energy is delivered to processes requiring ATP (contraction and ionic homeostasis) (105). The coupling of electron flow to ATP synthesis is referred to as oxidative phosphorylation.

ROS production

Recent findings indicate that 0.15% of electron flow that is normally reduced by complex IV to reduce O_2 to H_2O is converted to the superoxide anion ($O_2^{-\bullet}$) (112). Superoxide anion production occurs by the constant slow transfer of electrons onto O_2 from complex I (118) and the semiquinone radical located in complex III (117). ROS can have an unpaired electron: the superoxide anion, which is dismuted to hydrogen peroxide (H_2O_2), is an example. ROS include active metabolites of oxygen such as H_2O_2, which reacts with divalent cations in the Fenton reaction, forming the very reactive hydroxyl radical (OH•) [see review (46)]. The catalase reaction decomposes H_2O_2 back to H_2O and O_2. In the cardiomyocyte, an antioxidant system maintains ROS at very low levels through the combined action of nonenzymatic (*e.g.*, vitamin E, vitamin C) (47, 54) and enzymatic (superoxide dismutase, glutathione peroxidase/glutathione reductase system, and catalase) (4, 29, 48, 103) antioxidants.

Apart from being a by-product of oxidative phosphorylation, ROS are of considerable physiologic interest, regulating or participating in cellular events such as mitogenic and hypertrophic processes (104, 125), apoptosis, and necrosis after reperfusion injury and heart failure, as well as participating in the activation of preconditioning, a cellular stress response that blunts injury in response to oxidative stress (16).

MITOCHONDRIAL ROS EXCITABILITY

Mitochondrial ROS generation is an autocatalytic process: mitochondria respond to elevated ROS in the environs by dramatically enhancing their own ROS production. For example, the addition of *tert*-butyl hydroperoxide to hepatocytes increased mitochondrial ROS production (93), and after an initial ROS generation by gamma irradiation of a carcinoma cell line, there occurred a progressive increase of ROS originating from the electron transport chain (80). Mitochondrial ROS excitability may be related to activation of the mitochondrial permeability transition pore (MPTP), as secondary ROS production was slowed by treatment with cyclosporin A (CsA), an inhibitor of the MPTP (53).

Inducing and imaging RIRR

Mitochondrial ROS excitability, or RIRR, was first explored at the level of the single mitochondrion using advanced techniques in fluorescence imaging. Huser and Blatter (19) developed a method for generating ROS in the matrix space of isolated cardiomyocyte mitochondria by loading them with tetramethylrhodamine ethyl ester (TMRE), a fluorescent dye that accumulates in mitochondrial matrix according to membrane potential ($\Delta\Psi_m$) (19), and subjecting them to localized photoexcitation via laser scanning confocal imaging. They found that TMRE photoactivation caused mitochondrial depolarization, detected as a sudden decrease in TMRE fluorescence, which could be prevented by the addition of ROS scavengers. Dual loading of mitochondria with TMRE and calcein, the release of which serves as a marker for MPTP activity (94), revealed that the loss of mitochondrial TMRE fluorescence was followed by a slower ($t_{1/2}$ ~5 s) efflux of calcein. The irradiation-induced loss of TMRE was sensitive to CsA treatment, leading to the conclusion that the decrease in $\Delta\Psi_m$ reflected the opening of the MPTP (63).

Subsequently, Zorov *et al.* (126) applied tetramethylrhodamine methyl ester (TMRM) photoactivation to isolated cardiomyocytes using confocal line-scanning; an imaging mode in which the X-axis is continuously scanned at a fixed Y position (in this case, a longitudinal row of several mitochondria). The image obtained contains mitochondria in the X-axis (asterisks, Fig. 1b), and the Y-axis represents a time readout. The line-scanning imaging mode can be used to monitor $\Delta\Psi_m$ and ROS production simultaneously, and intensity values can be extracted to quantify changes at a subsecond resolution

(Fig. 1c). Zorov *et al.* found that at the level of the single mitochondrion, TMRM photoactivation yielded a short-lived (~5 s) burst of ROS accompanied by the collapse of $\Delta\Psi_m$. Although this phenomenon was insensitive to CsA, bongkrekic acid, an inhibitor of the MPTP, blocked both $\Delta\Psi_m$ collapse and the mitochondrial ROS burst, and $\Delta\Psi_m$ collapse was accompanied by mitochondrial inner membrane permeabilization to calcein, suggesting MPTP mediation of RIRR. We refer to this phenomenon as MPTP-RIRR (126).

More recently, a second mode of RIRR was described, which is independent of the MPTP. Two-photon excitation of TMRE localized to a small number of mitochondria in the cardiomyocyte resulted in cell-wide oscillations of $\Delta\Psi_m$ and ROS production. This phenomenon was bongkrekic acid sensitive, yet insensitive to CsA, and did not result in permeabilization of the mitochondrial inner membrane to calcein, indicating a lack of MPTP participation. Aon *et al.* (6) found that this mode of RIRR was sensitive to ligands of the mitochondrial benzodiazepine receptor (mBzR) known to block inner mitochondrial membrane anion channels (IMAC). We refer to this phenomenon as IMAC-RIRR.

The mitochondrial generation of ROS during the depolarization phase may seem counterintuitive. It is well understood that mitochondrial ROS production by both complexes I and III can depend on the maintenance of a high $\Delta\Psi_m$; a small $\Delta\Psi_m$ decrease can result in significant attenuation of ROS production (9, 39, 72, 78). Complex I superoxide anion production occurs mainly by reverse electron transport and depends on a highly reduced state of mitochondrial $NAD(P)^+$ (76), a high proton-motive force (specifically ΔpH) and secondarily on a reduced Q-pool (78). In complex III, due to the high local electric force that results from high transmembrane electrical potentials, the escape of the semiquinone radical from its binding sites, and subsequent reaction with O_2, may be enhanced at high membrane potential and reduced when membrane potential is lowered. Hence, decreased $\Delta\Psi_m$, due to either increased H^+ backflow through the F_1F_0 ATPase stimulated by ADP (state 3 respiration) or the uncoupling of electron flow from ATP synthesis by increased proton permeability of the inner mitochondrial membrane, can reduce ROS production at complex I by decreasing $NAD(P)H/NAD(P)^+$(76) and possibly by decreasing the life span of the semiquinone radical. Conversely, at complex III, mathematical modeling indicates that with decreasing redox potential, the Q pool shifts from predominantly the Q form through a mixture of all three forms to strictly the QH_2 form, with the amount of the semiquinone radical peaking in between. Similarly, depending on ambient redox potential and membrane potential, the lifetime of the semiquinone radical should be expected to be a bell-shaped function of membrane potential. When both membrane potential and redox potential vary, more complex variations may occur (40, 76). Indeed, further mathematical modeling indicated that the brief increase in superoxide anion production during IMAC-RIRR may be the result of increased respiratory electron flux due to the collapsing $\Delta\Psi_m$ (34). This could temporarily increase the concentration of single-electron carriers able to react with O_2, and may extend to MPTP-RIRR. A ROS-activated vicious cycle of ROS production at complex I (73) or III (39), complex III–derived ROS destabilization of the MPTP (8), or efflux of respiratory substrates due to the MPTP and concomitant inhibition of respiration and an increase in local O_2 concentration, may also contribute to the burst of ROS.

ROS production after MPTP activation requires the presence of NADH (13), probably to maintain a sufficiently negative redox potential of the relevant electron carriers. The oxidation of NADH was observed during the ROS burst phase of RIRR (6, 126). The capacity of a mitochondrion to continue to produce ROS via either mode of RIRR is therefore limited by the rate of regeneration of NADH. Enzymes of the TCA cycle that generate NADH (source for RIRR electrons) are reversibly inactivated by ROS (95). PKCδ-dependent inactivation of pyruvate dehydrogenase limits regeneration of NADH (32) as would a reduction in the intramitochondrial concentration of pyruvate. ROS production would also cease if the mitochondrial membrane potential decreases below a critical level. Thus, once RIRR is engaged, the individual mitochondrial contribution of ROS is limited to bursts lasting ~5 s (6, 24, 126). Mitochondrial recovery of $\Delta\Psi_m$ and NADH follows restoration of TCA cycle activity to preburst levels. Recovery of TCA activity may occur after a decrease in oxidative conditions and requires desphosphorylation of the pyruvate dehydrogenase subunit E1 (32). Such a recovery has been observed during rapid oscillations (6) and after lag times of several minutes (24).

Adult cardiomyocytes contain thousands of electrically independent mitochondria, arranged as a grid (Fig. 1a). This unique spatial organization allows the investigation of local versus global mitochondrial dysfunction. In this experimental model, we and others sought to determine the effect of a local mitochondrial perturbation on the entire cellular mitochondrial population. Using laser scanning confocal microscopy (24) and two-photon laser scanning microscopy (6), small numbers of TMRM/E-loaded mitochondria were subjected to photoactivation. In both cases, mitochondrion-to-mitochondrion transmission of RIRR was observed. However, the diverging results discussed later indicate that two modes of RIRR exist in the cardiomyocyte.

PORE MEDIATORS OF RIRR

Intracellular generation of mitochondrial ROS, by both IMAC-RIRR and MPTP-RIRR, is emerging as an important mediator of I/R injury in the heart. However, definite differences exist between the two modes of RIRR. These differences are linked to the molecular participants and give additional insight into ROS signalling during I/R injury. We discuss the components governing the two modes of RIRR and their links to the pathophysiology of I/R injury.

A mode of RIRR mediated by the mitochondrial permeability transition pore

Early experiments involving RIRR established a link with the MPTP (62, 126). The MPTP is a nonspecific pore that spans both the inner and outer mitochondrial membranes. Under conditions of enhanced matrix Ca^{2+} levels and/or oxidative stress, its activation results in IMM permeability to solutes up to 1.5 kDa (36). Moreover, MPTP activation can

lead to matrix swelling and release of cytochrome *c* into the cytosol, thereby activating apoptosis. Cytochrome *c* release as a result of MPTP activation is distinct from recruitment of proapoptotic Bcl-2 family members, which permeabilize the outer mitochondrial membrane (OMM) by a mechanism insensitive to CsA (45). MPTP activation may amplify any cellular energetic crisis that may have preceded the ROS insult, as mitochondria become ATP consumers, via ATP hydrolysis and reverse proton pumping by H^+-ATPase (85), a process that should be limited by the inhibitor subunit IF1 of the H^+-ATPase.

It is thought that the minimal assembly that can constitute the MPTP is composed of the voltage-dependent anion channel (VDAC), the adenine nucleotide translocator (ANT), and cyclophilin D (Cyc-D) (35). Cyc-D is a peptidylprolyl isomerase and belongs to a class of chaperones. At present, its function other than its participation in the MPTP is unknown, and the peptidylprolyl isomerase activity of Cyc-D is not required for its function in the MPTP (11). VDAC and ANT are apparently multifunctional. Under normal conditions, VDAC is in the OMM and forms a pore of about 3 nm, permeable to medium-sized ions [$\leq$5 kDa (87)], and the ANT is responsible for ADP/ATP translocation across the IMM and is thus essential for ATP delivery to the cytosol. Inhibition of the MPTP with CsA has been shown to prevent cardiomyocyte cell death caused by I/R (21). Recently, *in vivo* studies in Cyc-D–null mice have confirmed the MPTP, or at least Cyc-D, as a mediator of I/R injury in the heart (11, 12, 92). However, as mitochondria from ANT-null mice still exhibit MPTP activity (71), the conditions under which the ANT participates in the mitochondrial permeability transition are still unclear.

ROS are considered potent activators of the MPTP. In view of their relatively low concentrations, it is unlikely that they act through oxidizing the bulk of GSH, NADPH, and/or NADH. ROS may rather oxidize enzyme bound forms of these compounds and mediate MPTP activation through the oxidation of sensitive sulfhydryl residues of MPTP constituents (31). Sulfhydryl groups of the ANT may be targets of ROS. The cross-linking of specific thiol groups has been shown to stabilize the "c (cytosolic)" conformation of the ANT, which is also stabilized by MPTP activators carboxyatractyloside and palmitoyl-CoA. While enhancing Cyc-D binding to the ANT presumably in its "c" form, and antagonizing ADP binding, this results in enhanced sensitivity of the MPTP to Ca^{2+} (89). Cytosolic ATP, ADP, and bongkrekic acid enhance the alternative "m" conformation and inhibit the MPTP (53). It is the "c" conformation of the translocator that interacts with VDAC, an interaction that also engages cytochrome *c* (122). Cyc-D might catalyze the rather profound conformational transition that may be required for the ANT to change from the "c" form to a high-conductance nonspecific pore.

Although calcium (Ca^{2+}) is considered an important mediator of MPTP activity, investigations consistently show that neither the MPTP-mediated nor the MPTP-independent RIRR are dependent on Ca^{2+} (6, 24, 126). These results highlight the importance of ROS regulation of mitochondrial homeostasis and indicate that acute mitochondrial injury with disruption of Ca^{2+} homeostasis may be a secondary phenomenon. This opinion is supported by Zorov *et al.* (126), who demonstrated that Ca^{2+} sparking increased after RIRR induction (126). Likewise, Lemasters' group (69) used high spatial and temporal imaging to show that in adult cardiomyocytes after simulated I/R, Ca^{2+} entry into the mitochondria is the result, rather than cause, of ROS-mediated MPTP activation.

I/R injury and MPTP-RIRR

Several independent studies have demonstrated that RIRR is linked to hypoxia/reoxygenation–activated pathways of cell death, via the MPTP. CsA, a classic inhibitor of the MPTP, and sanglifehrin, a recently described specific inhibitor of the MPTP (33), both reduced infarct size after I/R in rat hearts (33) and also reduced the responsiveness of the MPTP to phototriggering via confocal imaging of the entire cardiac cell (57, 66). Although Zorov *et al.* (126) reported that RIRR was insensitive to CsA, the technique used by Duchen's group generates less photooxidative stress over time than does confocal line-scanning, as portions of the cell are scanned less frequently. This supports the notion that CsA inhibition of the mitochondrial permeability transition is a graded phenomenon (63). Importantly, CsA and sanglifehrin both protected against I/R and retarded RIRR induction in isolated human atrial cardiomyocytes, indicating the importance of RIRR signaling for understanding human I/R injury (107). Notably though, mitochondrion-to-mitochondrion transmission of RIRR was blocked by CsA, indicating that endogenous RIRR generates less oxidative stress than does TMRM photoactivation via sufficiently long periods of line-scanning (24).

Preconditioning and MPTP-RIRR

MPTP-RIRR may be linked to preconditioning, the phenomenon by which a transient bout of I/R can significantly protect the myocardium against a subsequent prolonged I/R insult (91). PKCϵ, the mitochondrial ATP-sensitive potassium channel (Mito-K_{ATP}) (86), and reactive oxygen species (119) are considered key mediators of preconditioning.

Juhaszova *et al*. (66) found that preconditioning, via brief hypoxia/reoxygenation, phorbol 12-myristate 13-acetate (PMA; PKC activator), or diazoxide (a putative activator of mito-K_{ATP} channels), decreased mitochondrial RIRR sensitivity, whereas prolonged hypoxia, followed by reoxygenation sensitized mitochondria to RIRR induction. These results indicate that the sensitivity of mitochondrion to undergo RIRR may be decreased via preconditioning protocols that are tuned to the kinetics of some of the regulatory processes involved. The authors discovered that the most proximal mediator of RIRR is glycogen synthase kinase-3β (GSK3β). GSK3β under normal conditions is active and is inactivated by phosphorylation at serine 9. GSK3β inhibition during I/R has been shown to be cardioprotective via phosphatidylinositol-3 kinase (116) and mTOR (52). Juhaszova *et al*. found that inactivation of GSK3β, through pharmacologic agents and RNAi, increased the time needed to induce RIRR via TMRM photoactivation, reflecting an elevated threshold for induction of the MPTP. Taken together, these results indicate that GSK3β is a point of convergence for the protective kinase cascades, and their result is to decrease MPTP sensitivity to oxidative stress. It is notable, though, that RIRR sensitivity

was not increased in constitutively active GSK3β S9A mice, suggesting that GSK3β is relevant primarily as a target for protective pathways (66).

A mode of RIRR mediated by the mitochondrial benzodiazepine receptor and mitochondrial inner membrane anion channel

Mitochondrial benzodiazepine receptor. The mitochondrial benzodiazepine receptor (mBzR; also known as the peripheral benzodiazepine receptor) has emerged as a mediator of a mode of RIRR (1, 6) and as an important target for protecting against mitochondrial dysfunction and I/R injury (1, 82). The mBzR is a multimeric complex consisting of multiple molecules of an 18-kDa isoquinoline carboxamide binding protein (IBP), which interact with VDAC (both in the OMM) and ANT (in the IMM) at contact sites (88). Although VDAC and ANT are also putative components of the MPTP, the mBzR can be distinguished from the MPTP through functional consequences of ligand interactions, as discussed later. Little is known concerning the role of mBzR, and the field has largely been advanced using pharmacologic agents (see Table 1), where tudies indicate a role in steroid biogenesis, regulation of VDAC, and mitochondrial respiration (120).

Ligand studies have shown that the mBzR plays a role in ROS-mediated cell (dys)function, both in regulating the generation of mitochondrial ROS and in the response to oxidative stress (Table 1). The expression of the IBP component of the mBzR resulted in increased susceptibility to H_2O_2-induced cell death in Jurkat cells (27), whereas knockdown of IBP

Table 1. Effects of mBzR Ligands on Cell and Organ Physiology

Synthetic	*mBzR ligands*	*Protective effect*	*Detrimental effect*
PK11195	1-(2-chlorophenyl)-*N*-(1-methylpropyl)-3-isoquinoline-carboxamide	Blocked RIRR waves (6) in isolated rat cardiomyocyte4s.	Activated the MPTP in rat heart tissue (30).
		Blocked loss of $\Delta\Psi_m$ in perfused rat heart (1), but failed to attenuate ECG changes with ischemia, in humans (41)	Activated the MPTP in L929 fibroblasts (102 and cerebellar granule cells (64).
			Blocked SSRI80575-mediated protection from H_2O_2 in H9c2 cardiac cells by (82)
			Decreased function in hanging rat heart (43)
Ro5-4864	4′-chlorodiazepam	Blocked TNF-α induced cell death in U937 lymphoblastoid cells (20)	Decreased function in hanging rat heart (43)
		Protected against H_2O_2-activated injury in H9c2 cells (82)	
		Blocked loss of $\Delta\Psi_m$, AP shortning, and arrhythmias in perfused rat heart (1)	
SSR180575	7-chloro-*N,N,*5-trimethyl-4-oxo-3-phenyl-3,5-dihydro-4H-pyridazino[4,5-b]indole-1-acetamide	Protected against H_2O_2 activated cell death in Jurkat cells (27)	
		Protects against renal ischemic injury in vivo. Limits ROS production in cell model (75)	
		Protected against H_2O_2-activated injury in H9c2 cells and reduced infarct size in isolated rat and in vivo rabbit hearts after I/R (82)	
FGIN-1-27	*N,N*-di-n-hexyl 2-(4-fluorophenyl)indole-3-acetamide		Potentiated TNF-α induced cell death in L929 fibroblasts (102)
			Sensitized to ROS-activated $\Delta\Psi_m$ collapse in rat cardiomyocytes (6)
			Increased I/R-induced myocardial dysfunction in perfused rat (1)

ECG, electrocardiogram; $\Delta\Psi_m$, mitochondrial membrane potential; I/R, ischemia/reperfusion; MPTP, mitochondrial permeability transition pore; mBzR, mitochondrial benzodiazepine receptor; ROS, reactive oxygen species; RIRR, ROS-induced release.

was associated with increased tumorigenicity and apoptosis resistance (84). Treatment of rats with SSR180575 was shown to limit ROS production and cell death *in vivo* and to protect against exogenous H_2O_2 in a cell-culture model (75). mBzR may also play an important role in the response to oxidative stress in the heart. SSR180575 and Ro5–4864 both blocked H_2O_2-mediated cell death in the H9c2 cardiac cell line. Furthermore, SSR180575 reduced contractile dysfunction and infarct size in an isolated perfused rabbit heart model, as well as in an *in vivo* rat model of I/R injury (82). It is noteworthy that in this study, PK11195, a competitive inhibitor, was found to block SSR180575-mediated protection against H_2O_2 in H9c2 cells.

Although PK11195 has been shown to potentiate programmed cell death, as well as activate the mitochondrial permeability transition pore in cardiomyocytes (30), recent findings indicate that its prodeath activity may be independent of the mBzR (50). As shown in Table 1, results obtained with mBzR ligands can be confusing, as some ligands are considered agonists, whereas others are antagonists, yet under certain circumstances, both can have similar physiologic effects (28). Such ambivalent behavior has also been observed for opiate-receptor ligands (110). PK11195 did not protect against simulated ischemia in human *in vivo* (41) or in isolated rat hearts. Both Ro5 4864 (10 μ*M*) and PK11195 (100 n*M*) were found to decrease function significantly in the working heart (43).

Because the mBzR includes the ANT and VDAC, it is tempting to attribute its role in RIRR to effects on the MPTP. We discuss the evidence that mBzR is involved in a mode of RIRR that does not involve the MPTP, referred to as IMAC-RIRR.

Mitochondrial inner membrane anionic channels

The mBzR may modulate the activity of the inner mitochondrial membrane anion channel (IMAC), and it has been proposed that it may mediate a second mode of RIRR (IMAC-RIRR). The IMAC is permeable to a range of anions (conductance of ~100 pS (70, 111) and is thought to be involved in maintaining volume homeostasis (14) and regulating superoxide anion release from the mitochondrial matrix (119). The IMAC has been shown to be blocked by DIDS (15), and at nanomolar concentrations, the mBzR ligands PK11195 [which binds the IBP component of the mBzR (65)] and Ro5–4864 [which may (88) or may not (65) require VDAC for binding to the IBP] have been shown to inhibit IMAC activity in heart mitoplasts (70).

IMAC-RIRR and I/R injury

The O'Rourke group has reported that after localized photoactivation of TMRM, cell-wide $\Delta\Psi_m$ oscillations (and waves of $\Delta\Psi_m$ collapse) and concurrent bursts of mitochondrial ROS occurred in adult cardiomyocytes. Importantly, this phenomenon was insensitive to CsA, and permeability of the inner mitochondrial membrane to calcein was not observed. However, this mode of RIRR was sensitive to mBzR ligands, including PK11195, which was reported not to affect MPTP-RIRR (66). Recently this group has elucidated the pathophysiologic significance of IMAC-RIRR during early I/R injury in the hanging heart.

Sarcolemmal ATP-sensitive potassium (K_{ATP}) channels are linked to the cellular energy state, being activated by decreases in cytosolic ATP concentration. At the level of the single cell, mitochondrial uncoupling, and consequent ATP decrease, is sufficient to activate sarcolemmal K_{ATP} channels (106). RIRR-triggered cell-wide $\Delta\Psi_m$ oscillations were found to parallel changes in sarcolemmal K_{ATP} activity, with the mitochondrial depolarization phase coinciding with increased K_{ATP} current (and action-potential shortening), and the mitochondrial repolarization phase coinciding with decreased K_{ATP} activity (and action-potential recovery) (6). Most important, in this report, the $\Delta\Psi_m$ oscillations were CsA insensitive, yet prevented by blocking the mBzR with PK11195. Conversely, FGIN-1–27 sensitized mitochondria to $\Delta\Psi_m$ collapse in isolated adult cardiomyocytes (6). This suggests the involvement of mBzR, but now together with IMAC rather than with ANT.

The potential implications of IMAC-RIRR–dependent mitochondrial dysfunction in the context of myocardial function during I/R injury were subsequently investigated. Similar to the finding in the isolated cardiomyocyte, PK11195 and 4'-Cl-DZP were found to block early I/R injury (specifically shortened action-potential duration and arrhythmia) in the isolated heart, whereas FGIN-1–27 amplified myocardial dysfunction (1). As such, maintaining mitochondrial function under conditions in which ROS production is increased, and thus IMAC-RIRR sensitivity is increased, at the organelle level serves to maintain ATP supply and prevent myocardial dysfunction at the organ level.

The results establish the existence of a mode of RIRR that is independent of MPTP, involves the IMAC (IMAC-RIRR), and is linked to early events in I/R injury. The extent to which the two RIRR processes are truly independent is uncertain; they may both involve the IBP, which might associate either with IMAC or with VDAC-ANT, or perhaps even with both. Although DIDS and PK11195 were reported not to affect photoactivated MPTP-RIRR at the level of single mitochondrion RIRR (66), DIDS suppressed MPTP-RIRR wave generation (24). It remains to be determined whether mBzR inhibition will affect mitochondrion-to-mitochondrion transmission of MPTP-RIRR.

It is possible that RIRR is accomplished through a network of pathways rather than a single pathway, where both parallel and serial modes may be involved. Such a mechanism may be understood only in terms of control analysis (61) and through systems biology approaches (123).

WHICH ROS ARE INVOLVED?

It appears that the different modes of RIRR are governed by different ROS; although the precise specificities have not been established, some clues exist.

Topology

Rotenone, an inhibitor of complex I, blocked IMAC (6) and MPTP-mediated (126) (13) ROS production, demonstrating the requirement in these experiments for electrons entering at complex I for both modes of RIRR. Although the site(s)

in the electron transfer chain where ROS emerges in the genesis of RIRR has not been entirely determined, experiments using myxothiazol and antimycin A indicate that complex III of the ETC participates in IMAC-RIRR (6) and MPTP-RIRR (24). Of note is that we found that antimycin A blocked laser-induced depolarization and ROS production (unpublished results), whereas in the case of IMAC-RIRR, antimycin A potentiated $\Delta\Psi_m$ oscillations. Further studies are needed to explore this potentially important mechanistic distinction. For both MPTP- and IMAC-RIRR, early ROS production in the mitochondria was detected using dichlorodihydrofluorescein (H_2DCF) derivatives, which localize to the mitochondrial matrix (6, 114). Although H_2DCF is insensitive to the superoxide anion, it is sensitive to H_2O_2 (81), which is immediately generated on spontaneous or catalyzed dismutation. As such, DCF results indirectly indicate that matrix-side superoxide anion production is common to both modes of RIRR. Although this suggests the matrix as the site of first ROS production, this is paradoxical by itself, as it is likely that ROS arise from the reduction of O_2 in the usual triplet or perhaps the less common singlet form by single-electron carriers of low midpoint potential, such as semiquinone radical (25). Most of the latter would reside in the membrane, making the membrane the more likely site of primary production of ROS. Local permeability of the matrix and the intermembrane phase of that membrane may then determine where the superoxide anion and its immediate products end up. This presents the possibility that before moving to the aqueous phases bordering the membrane, ROS could affect the ANT or IMAC.

Exiting the mitochondria

The charge of the superoxide anion is likely to impede its crossing the hydrophobic mitochondrial inner and outer membranes. Conversely, that same negative charge should help its expulsion through anion channels, including the ANT and IMAC, and VDAC in the outer membrane. A transmembrane electric potential of 150 mV would enhance its movement by a factor of ~700 (124). Release of ROS to the cytosol appears to be involved in RIRR transmission: DIDS was found to inhibit both modes of mitochondrion-to-mitochondrion transmission of RIRR (6, 24). Although DIDS has been used to target IMAC (5, 74), DIDS also inhibits VDAC, which appears at least partially to govern superoxide anion egress across the outer mitochondrial membrane (55). The effect of DIDS demonstrates that compartmentalization of superoxide anion governs propagation to nearby mitochondria, regardless of the mode of RIRR. The precise manner by which ROS exit the mitochondrion, be it via VDAC *or* IMAC, remains unclear. Although DIDS blocks both modes of RIRR propagation, it does not block local, photoactivated RIRR, possibly because of the magnitude of the ROS burst after photoactivation.

Signaling ROS

Under normoxic conditions, intracellular oxygen concentration in the cardiomyocyte is in the 3- to 8-μ*M* range (22), with steady-state mitochondrial H_2O_2 concentrations at 5 n*M* and superoxide anion concentrations at around 0.1 n*M* (26). Superoxide anion dismutation is limited by the diffusion rate of superoxide anion to SOD (49), and as such, the superoxide anion is extremely short-lived, with a half-life of 10^{-4} s (10). Yet, electrophoresis of the superoxide anion through a pore at high membrane potential could well be faster than this.

Experimental (6) and theoretical (34) work indicates that IMAC-mediated RIRR may involve superoxide anion as a second messenger. The authors calculated that at nanomolar superoxide anion concentrations, its turnover should be within ~1 s. They concluded that micromolar superoxide anion concentrations are necessary to propagate IMAC-RIRR. Indeed, the time frame for coordination of the population of mitochondria agrees with superoxide anion diffusion dynamics (7) and the superoxide dismutase mimetic TMPyP blocked RIRR oscillations (6). Finally, the differences in ROS are reflected in the kinetics of RIRR transmission. IMAC-RIRR waves were measured at 22 μm/s, which is consistent with superoxide anion functioning as the second messenger (7).

However, the slower MPTP-RIRR wave velocity indicates a longer-lived ROS than superoxide anion, or some other, longer-lived signal between the mitochondria. Superoxide dismutase mimetics did not alter RIRR, yet broad-spectrum antioxidants vitamin E (lipophilic antioxidant) and Trolox (water-soluble version of vitamin E) blocked RIRR and RIRR transmission (24, 126). Moreover, $DCFH_2$ (81) and BODIPY $C11^{(581/591)}$ (101), which are insensitive to the superoxide anion but sensitive to peroxides, were highly oxidized in our experiments involving mitochondrion-to-mitochondrion transmission of RIRR, which was independent of laser-activated RIRR (24) (Fig. 2). MPTP-mediated RIRR might involve a downstream peroxide or an oxidized lipid (77).

DISCUSSION AND PERSPECTIVES

ROS-induced ROS release (RIRR) is perhaps only one of the processes that govern the dynamics and extent to which the mitochondrial population of a heart cell participates in ROS production. However, it appears to be an important one, and it connects the phenomenon to one of the major function of healthy cells (*i.e.*, oxidative phosphorylation producing ATP). For I/R injury, the ETC serves as the source of ROS (2) with a requirement for redox pressure at complex I (17). This is true for both IMAC and MPTP-mediated RIRR (6, 24, 126). Enhanced ROS production, a key cause of I/R injury (16), may well be mediated by both IMAC- and MPTP-RIRR.

Mitochondrial density is a double-edged sword in the cardiomyocyte. Massive mitochondrial content [>30% (98)] is necessary to maintain ATP levels sufficient to support contractile function. Yet mitochondria also behave as an excitable medium: local perturbations to mitochondria can result in cell-wide disturbances. This is an important concept, as the apoptotic response in the cell is coordinated at the subcellular level via intermitochondrial communication (97). Recently, mitochondrial size and placement within the cell were shown to be determinants in mitochondrion-to-mitochondrion transmission of apoptotic signals (115). Although one continuous mitochondrial reticulum would enable power-sharing throughout the cardiomyocyte (109), a focal apoptotic event

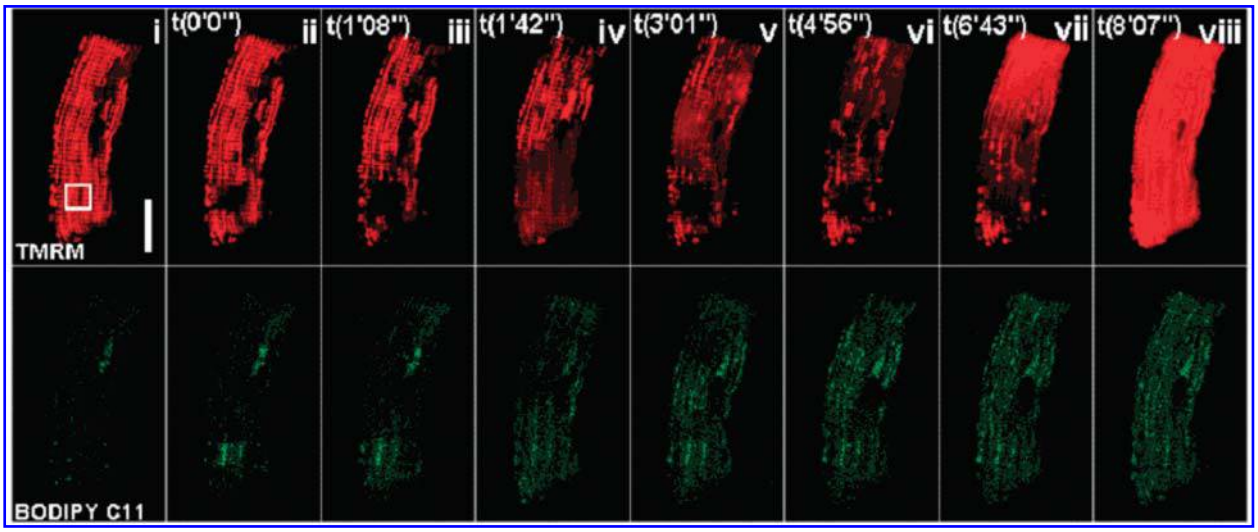

FIG. 2. RIRR wave of depolarization coincided with increased lipid peroxidation. Rabbit cardiomyocytes were dual loaded with 0.5 μ*M* BODIPY C11$^{(581/591)}$ and 0.2 μ*M* TMRM and imaged by confocal microscopy. The presence of ROS was detected with the lipophilic, red fluorescent dye, BODIPY C11$^{(581/591)}$. This probe embeds into membranes, and on oxidation, undergoes red-to-green shift and can therefore be used as a marker for lipid peroxidation (Pap et al., 1999). The cell was scanned (543 nm) in the region bordered by the white box (i) until mitochondria locally depolarized. After localized production of ROS due to photoexcitation of TMRM, a wave of depolarization proceeded at a velocity of 5 μm/min (ii–vi). The mitochondrial depolarizations (indicated by loss of red fluorescence) coincided with increased green BODIPY C11$^{(581/591)}$ fluorescence [*i.e.*, increased lipid peroxidation (ii–vi)]. After the wave of depolarization, the mitochondrial population repolarized at a velocity of 40 μm/min (vii–viii). Scale bar, 20 μm. Reproduced with permission from (24). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars.)

could lead the entire cell to programmed cell death. Thus, it is possible that the independent nature of cardiomyocyte mitochondria may, up to a certain threshold, allow for tolerance toward a subpopulation of malfunctioning (RIRR generating) mitochondria.

The existence of two distinguishable modes of RIRR indicates that mitochondrial ROS production is a complex process. Clearly, though, the tendency for an individual mitochondrion to undergo RIRR is strongly linked to factors that either promote or prevent cell death. IMAC-RIRR governs early electrical dysfunction in the reperfused heart. Conversely, MPTP-RIRR is linked to downstream death pathways as it is inhibited by the reperfusion injury salvage kinase (RISK) pathways, which can protect against I/R injury [*e.g.*, insulin-dependent pathways (37), the mito-K_{ATP} channel (58), and PKC activation (66)]. These results argue that MPTP-mediated RIRR may serve as the amplification mechanism to create lethal ROS levels after I/R. Downstream of RIRR activity are PKCϵ (66), Bax translocation (38), hypercontraction (24, 60), and possibly p38 activation (74). These findings also demonstrate that RIRR sensitivity is a readout that may help identify upstream targets that prevent mitochondrial dysfunction, both in the isolated cardiomyocyte and hanging heart model.

RIRR may transition from IMAC- to MPTP-mediated RIRR. This would be supported by the finding that CsA did result in some protection, although less than that provided by IMAC inhibitors in one study (1). Work by the O'Rourke group has shown that DIDS and mBzR ligands influence RIRR. However, the mBzR ligand Ro5–4864 modulates both IMAC and the MPTP at the Ro5–4864 concentrations used (70), and DIDS is a known inhibitor of VDAC (55). This makes it impossible to distinguish satisfactorily between the IMAC and MPTP modes of RIRR at this moment: further investigation is required into the relation between RIRR and the ANT. Oxidative modification of the ANT may be a common triggering event for RIRR, as both modes of RIRR are bongkrekic acid sensitive (6, 126). It is possible that the IMAC-RIRR underlies a more excitable state, which occurs before and contributes to MPTP-RIRR. Such a transition was recently reported (60). We suggest that the feed-forward strength of MPTP-RIRR may then supersede IMAC-RIRR.

Because the RIRR appears to be an essential mediator of mitochondrial ROS production, it is noteworthy that the preconditioning phenomenon requires mitochondrial ROS production (119). Mito-K_{ATP} channel activation by diazoxide leads to a CsA-dependent, yet transient MPTP activation (67). In addition, recent work indicates that a transient activation of MPTP is required for preconditioning to occur (56).

Moreover, as opposed to IMAC-RIRR oscillations, we found that MPTP-RIRR–mediated mitochondrion-to-mitochondrion transmission was refractory; after a wave of depolarization, the mitochondrial population repolarized and was resistant to subsequent triggering of RIRR (Fig. 2). This result may demonstrate visually the preconditioning process, in which a primary wave of RIRR can activate preconditioning, including the desensitization of the MPTP.

Cardiac mitochondria are electrically independent and exhibit heterogeneous sensitivities to proapoptotic stimuli, including oxidative stress and calcium overload (99, 100, 126), as well as in mitochondrial oxidative-damage aging (113). A remarkable aspect of RIRR is the heterogeneous response of

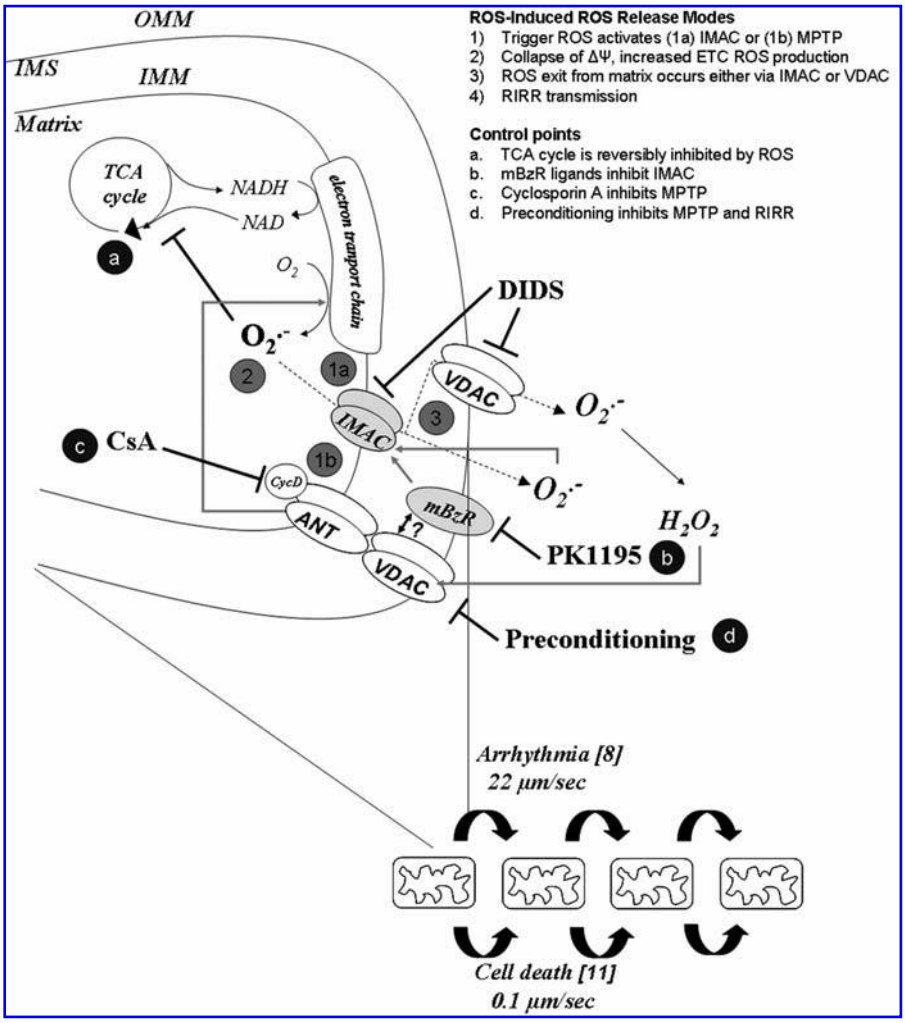

FIG. 3. Schematic of IMAC- and MPTP-RIRR.

individual cardiac mitochondria to oxidative stress in response to antimycin A, photoactivation of the MPTP (126), or after hypoxia/reoxygenation (66). Moreover, RIRR sensitivity increases with age (77). An underlying concept, then, is that the capacity for the cell's mitochondrial population to resist RIRR should be a major determinant of the cell's ability to withstand acute injury or aging. As such, identifying factors that contribute to increased sensitivity raises the possibility that dangerous mitochondria may be selectively removed. Mitochondria are known to be sequestered by a double-membrane vesicle (macroautophagosome), which then delivers the mitochondria to the lysosomes where they are degraded. MPTP activation has been shown to be one trigger for the removal of damaged mitochondria via this pathway (44), which may serve as a means to purify the cell's functioning mitochondrial population. This process has been termed mitophagy by Lemasters (83) and mitoptosis by Skulachev (108). It may be more useful to regard the destabilized mitochondria as undergoing mitoptosis, with the resultant autophagic removal as mitophagy. This process could well be a component of a systems biology mechanism by which the cell regulates a graded response to insults, which then may or may not turn into full-blown apoptosis.

Because of the imperfect specificity of small-molecule inhibitors, it will be necessary to use other ways of modulating the activity of RIRR components, such as RNAi and tunable promoters, as recently reported connecting GSK3β to RIRR using RNAi and knockout mice (66). Moreover, the recent development of a H_2O_2-sensitive GFP mutant will allow us to improve our understanding of subcellular and submitochondrial ROS production, compartmentalization, and signaling dynamics during RIRR (18). Finally, with the generation of both Cyc-D–null (11, 12, 92) and Cyc-D–overexpressing mice (11), we have now the opportunity to elucidate the control of MPTP over RIRR, as well as to explore differences between the two RIRR modes.

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ABREVIATIONS

ANT, adenine nucleotide translocator $\Delta\Psi_m$; Ca^{2+}, calcium; Cyc-D, cyclophilin D; CsA, cyclosporin A; ETC, electron transport chain; GSK3β, glycogen synthase kinase-3β; H_2DCF, dichlorodihydrofluorescein; H_2O_2, hydrogen peroxide; IBP, isoquinoline carboxamide–binding protein; IMAC, inner mitochondrial membrane anion channel; IMM, inner mitochondrial membrane; IMS, intermembrane space; I/R, ischemia–reperfusion; mBzR, mitochondrial benzodiazepine receptor; MPTP, mitochondrial permeability transition pore; OMM, outer mitochondrial membrane; O_2, molecular oxygen; RIRR, ROS-induced ROS release; ROS, reactive oxygen species; TMR, tetramethylrhodamine ethyl ester; TMRM, tetramethylrhodamine methyl ester; TCA, tricarboxylic acid; VDAC, voltage-dependent anion channel calcium.

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