MITOCHONDRIAL REACTIVE OXYGEN SPECIES (ROS) AND ROS-INDUCED ROS RELEASE

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I. INTRODUCTION

Photo-activated reactive oxygen species (ROS) may trigger mitochondrial permeability transition pore (mPTP) induction within individual mitochondria in intact cell systems. The phenomenon of ROS-triggering of the mPTP associated with further stimulation of ROS formation has been termed “ROS-induced ROS release” (RIRR) (491). mPTP opening is a mitochondrial response to an oxidative challenge resulting in an amplified ROS signal, which depending on ROS levels may result in different outcomes. In addition to ROS effects in those mitochondria (where the RIRR originated), ROS released into cytosol could trigger a complex cellular signaling response and/or RIRR in the neighboring mitochondria. In the latter case, ROS trafficking between mitochondria could constitute a positive-feedback mechanism resulting in an elevated production of ROS that could be propagated throughout the cell and may cause perceptible mitochondrial and cellular injury. Although photo-induced formation of ROS could be initially used in the experimental setting as a trigger for more massive, avalanche-like ROS release, this phenomenon is representing a more fundamental mechanism, e.g., light-independent spontaneous redox transitions associated with the induction of mPTP or other mitochondrial channel(s) that may occur under different physiological or pathological conditions with corresponding impacts on mitochondrial and cellular physiology. This review will cover the spectrum of RIRR-related phenomena, both physiological and pathological including the processes of mitochondrial ROS production...
and scavenging. Ultimately, the imbalance between the inflow, neutralization, and outflow of ROS with corresponding triggers in specific cell signaling pathways may result in extreme situations such as oxidative and reductive stresses with the consequent onset of numerous pathologies or even the cell and organismal death.

II. ROS: GENERAL DEFINITIONS

Eleven years ago this journal published an excellent and comprehensive review by Droge (117) on free radicals and their beneficial and detrimental roles in cell physiology and pathology. Since then, the general interest surrounding the roles of these species has constantly increased, shifting the main focus to highly potent oxidants containing oxygen, called ROS. The term ROS encompasses oxygen free radicals, such as superoxide anion radical (O$_2^-$) and hydroxyl radical (·OH), and nonradical oxidants, such as hydrogen peroxide (H$_2$O$_2$) and singlet oxygen (¹O$_2$).

ROS can be interconverted from one to another (depending on $\Delta G$ of relevant processes) by enzymatic and nonenzymatic mechanisms. The primary and most abundant ROS is the superoxide anion radical that has a comparatively high oxidative capacity [standard redox potential of the oxygen/superoxide couple $E^{\circ} = -0.137$ V (337)] allowing single-electron reduction of molecular oxygen by certain mitochondrial oxidoreductases. H$_2$O$_2$ is generated through spontaneous or superoxide dismutase (SOD)-catalyzed dismutation of O$_2^-$ (143). In mammals, three SOD isoforms were found in the living cell with precise compartmentalization: the Cu,Zn-dependent isoform (Cu,Zn SOD, SOD1) (142) is located in the mitochondrial intermembrane space and cytosol; the Mn-dependent isoform (Mn SOD, SOD2) (358, 468) is located in the mitochondrial matrix; and Cu,Zn SOD is located in the extracellular space (ecSOD, SOD3) (285).

The most potent and aggressive oxidant primarily responsible for oxidative damage of DNA bases is the hydroxyl radical, which has a relatively short half-life. OH can be generated through a variety of mechanisms. It is well known that OH is generated from H$_2$O$_2$ and O$_2^-$ which is catalyzed by iron ions through the Haber-Weiss reaction (169) with a specific case of Fe$^{2+}$-mediated decomposition of H$_2$O$_2$ [the Fenton reaction (130), reviewed in Ref. 237]. Ionizing radiation causes decomposition of H$_2$O$_2$, which also results in forming OH and hydrogen atoms. OH could be also generated by photolytic decomposition of alkylhydroperoxides (447).

In addition, a number of other oxygen-containing free radicals are capable of causing oxidation of essential cell components: nitric oxide (NO), peroxynitrite, lipid hydroperoxides (LOOH), alkoxyl radical (RO•), peroxy radical (OOF•), nitrogen-centered radical, sulfate radical (SO$_4^{2-}$) and metal-oxygen complexes. These radicals combined with the previously mentioned ROS form a large and important group of active redox agents playing critical role in a number of intra- and extracellular processes.

III. ROS: FROM SIGNALING TO PATHOLOGICAL

One of our specific aims is to give an overview of a spectrum of phenomena associated with different ROS serving a “signaling” role (discussed below) which is essential for a large number of biochemical reactions. In the following sections we address the significance of reductive and oxidative stress. Under physiological conditions, the balance between ROS generation and ROS scavenging is highly controlled. Depending on circumstances, regulated oxidative stress could initiate diverse cellular responses ranging from triggering signaling pathways involved in cell protection, initiating coordinated activation of mitochondrial fission and autophagy to optimize clearance of abnormal mitochondria and cells to protect spreading the damage to the neighboring mitochondria and cells (100, 117, 490). On the other hand, unregulated oxidative and reductive stresses could result in severe cellular damage, unwanted cell death, and consequently whole organ and organism failure (102, 242, 498). Therefore, adaptive physiological redox stresses, such as those occurring under the process of a programmed removal of damaged biological systems including mitochondria and other cellular components (“physiological”), must be differentiated from maladaptive unwanted (“pathological”) oxidative damage.

Under normal physiological conditions, ROS emission (essentially, production minus scavenging) was considered to account for ~2% of the total oxygen consumed by mitochondria (81). A recent measurement of ROS production in mitochondria with disabled antioxidant systems revealed values fluctuating from 0.25 to 11% depending on the animal species and respiration rates (21). A lower, as well as a higher, percentage may have deleterious consequences since ROS, when low, are unable to provide proper cellular functioning through regulation of a great number of biochemical reactions. When high, they are unable to provide a controlled regulation. It will require conditions when the flux of metabolic regulators of ROS level is finely tuned to respond to the cell demands with mitochondria playing a critical role. As for many cellular signaling elements, the principle “muetet nocem” (excess is harmful) may become a key element in a switch operating between “signaling” (meaning as survival-promoting which is physiologically required for renovating a biological component) and pathological (meaning undesired death-promoting) modes (216, 218, 219, 232, 491, 493, 497). For example, the ROS-mediated ignition of a death of cells designed for long-term use (postmitotic cells such as cardiac myocytes or neurons), i.e., occurring under severe ischemic conditions causing pa-
thologies such as myocardial infarct or stroke, under any conditions may be considered a pathological event. In other cases, apart from their pro-survival signaling role, ROS are apparently involved in a designated physiological function, eliminating unwanted mitotic cells or mitochondria and a significant rise of local ROS level within might be an efficient means to fulfill such functions. Futhermore, ROS have been shown to play a central role in regulation of the cell cycle progression (453). Whether the ROS burst can serve a signaling (survival) function will be discussed in a section describing the oscillatory behavior of mitochondria. For clarity, we will stay within definitions of signaling ROS as of those serving essential pro-survival functions and anti-survival functions including required elimination of unwanted cells (quality control, maintenance of function), while pathological ROS are considered as those causing oxidant-induced unwanted changes including unwanted cell death (loss of function).

IV. ROS: REDOX STRESS

There is an apparent heterogeneity in ROS levels and types when comparing different cells and organs (54, 173, 271, 342, 373, 401, 477). This is largely due to a heterogeneous distribution of activities of ROS producing and utilizing machineries (11, 78, 103, 290, 346). The general consensus is that overwhelming ROS production when not compensated for with their scavenging by endogenous antioxidants will lead to the rise of ROS beyond the “normal” or “physiological” threshold level. This results in a process conventionally called “oxidative stress.” Apparently, the definition of this widely used term (up to the year 2012 this term yields over 100,000 citations in PubMed) is quite broad and has “soft borders” considering the scenarios presented above. This is due to the fact that physiological levels and types of ROS in different tissues and in different parts of the same tissue under different physiological conditions are heterogeneous and highly dependent on the energy load that is met by the cell response. Even within the confines of a single cell, there are at least eight distinct organelar compartments (mitochondrial matrix, lysosomes, smooth ER/SR, rough ER, the Golgi, peroxisomes, the nucleus, the cytosol), each with its own redox poise (315). Accordingly, the term oxidative stress is often used in the literature in a very general term to define a state when the levels and types of oxidants in the cell or the organelle on average significantly exceed the ground/resting/steady-state level associated with normal homeostatic function. At the opposite end of the redox spectrum, when the reduced glutathione levels are too high, “reductive stress” occurs and demonstrates potentially detrimental consequences for the cell (154, 349). Within normal fluctuation of energy load, the productions of ROS and the ROS levels in mitochondria, cells, and the tissue are safe to perform normal activity (maintenance of function) of the particular biological system. ROS signaling and the role of ROS in vital cellular functions associated with cell prolifer-

ation, differentiation, migration, immune response, cell senescence and death, and number of inherited or acquired pathologies such as ischemia-related disease, atherosclerosis, neurodegenerative disease, malignant transformation, diabetes mellitus, rheumatoid arthritis, aging, etc., is described elsewhere (25, 117, 185, 203, 263, 348, 352, 497). However, under stress, when the ROS levels remain outside the normal range (either under conditions of enhanced antioxidative pathways associated with reductive stress or of those characterized by the rise of uncompensated ROS associated with oxidative stress), resultant instability of the redox environment may develop that could be harmful (unwanted loss of function) if it is not compensated by the feedback control mechanism.

In summary, considering these scenarios, both high levels of ROS (oxidative stress) and excessively low levels of ROS (reductive stress) are deleterious and apparently play a causative role in the pathologies caused by malfunctioning processes related to the dramatic change of redox environment. Underlying all these arguments, redox homeostasis seems to be a critical factor for normal functioning of the mitochondrion, cells, and organisms (174). Previously, it has been argued that the cell normally maintains cytosolic thiols in a highly reduced state, thus not supporting the existence of reductive stress (154). More recently, however, a profound increase of reduced glutathione concentration and the ratio of GSH/GSSG in cardiomyopathic animals carrying the R120G mutation in the aB-crystallin molecule was detected (349). The elevated level of reduced equivalents in these animals was accompanied by the augmented expression together with increased antioxidative enzymatic activity of glutathione peroxidase, glutathione reductase, and catalase, which supports the implication of deleterious reductive stress (349). The presence of reductive stress in yeast was also confirmed (440). A more precise term, redox stress, might be introduced reflecting both the incidence of oxidative and reductive stresses; however, this review is focused primarily on the circumstances related to oxidative stress.

V. ROS GENERATION IN MITOCHONDRIA

In 1961, Jensen was among the first investigators to demonstrate that mitochondria produce ROS (209). He observed that a small portion of the oxygen consumed by submitochondrial particles oxidizing NADH or succinate was converted to \( \text{H}_2\text{O}_2 \) since this consumption was catalase sensitive. Later, in 1972–1973, a classic, more general study, was done at the Johnson Research Foundation in Philadelphia by Britton Chance and co-workers (57, 58) who initiated the modern era of the mitochondrial ROS research. Since that time, scientists debated the physiological relevance of data obtained using “artificial” systems such as isolated mitochondria, inside-out submitochondrial particles, reconstituted respiratory complexes, and pure en-
zymes. According to the critics, these are not adequate systems to extrapolate data to the cell, organ, and organism levels (reviewed in Refs. 340, 480, 492). However, some counter-arguments support the relevance of these model systems. We are still lacking a detailed mechanistic knowledge of the architecture of mitochondrial ROS-producing systems such as of complex I or complex III and detailed insights on the mechanisms controlling their activities. We will make an attempt to partially address and clarify this scientific debate and to present the arguments in support of, and against, the importance and physiological relevance of those specific proposed mitochondrial ROS-producing components. The primary goal of this review is not a comprehensive coverage of this specific issue, but the background that needs to be addressed here to provide a basis for understanding those cases when an “innocent” molecular site (i.e., normally associated with moderate and physiological ROS production) becomes a “killer,” producing ROS levels leading to the destruction of the biological system (perhaps through some poorly understood amplification mechanism). Good reviews on the current mechanisms of ROS production in mitochondria are available elsewhere for the reader interested in a general background and for those interested in substantially detailed mechanistic depth (3, 13, 201, 202, 308, 397, 416).

A. Complex II

1. Under normal conditions

We begin this review of mitochondrial ROS-producing sites from complex II since succinate is a more frequently used oxidative substrate to explore the functioning of isolated mitochondria which became a classical object to study ROS production.

Complex II, succinate-ubiquinone oxidoreductase (EC 1.3.5.1), commonly known as succinate dehydrogenase (SDH), is a tetrameric iron-sulfur flavoprotein of the inner mitochondrial membrane and acts as part of the Krebs cycle and respiratory chain. SDH catalyzes the conversion of succinate into fumarate, yielding reduced equivalents in the form of reduced flavin adenine nucleotide (FADH$_2$). This is followed by a reduction of ubiquinone to ubiquinol. Mammalian Complex II, as well as that from yeast, harbors a covalently bound FAD, three iron-sulfur clusters, a b-type heme, and two quinone-binding sites termed Q$_p$ and Q$_a$, standing for proximal or distal sites correspondingly.

Typically, complex II is excluded from the list of potential candidates for important physiological contributors of ROS (347, 348, 358). It is partially due to fact that the succinate level in the tissue is low (in a range of hundreds of micromoles), while in in vitro experiments with isolated mitochondria millimolar concentrations are used. Hansford et al. (175) found that while H$_2$O$_2$ production can be detected in mitochondria oxidizing succinate in vitro at experimental ambient (5–10 mM) concentrations, they do not produce significant amounts of peroxide at low, more physiologically relevant succinate concentrations (175).

Some studies point to flavins (flavin adenine nucleotides, FAD) of SDH rather than other electron-carrying components (such as iron-sulfur clusters or quinones) as the site of autoxidation responsible for generating ROS (200, 293, 294). Others implicate ubisemiquinone and iron sulfur centers as these sites, although under normal and steady-state conditions these components are only partially reduced and short-lived (167, 192, 267), thus giving a low probability to transfer single electrons directly to oxygen.

2. Redox regulation of ROS production and redox buffering

During oxidation of succinate in isolated respiring mitochondria, electron flow can bifurcate forming direct (towards cytochrome oxidase) and reverse (toward NAD; rotenone-blocked) transport with the latter requiring energy input (79, 80, 187). The succinate-driven ROS generation during reverse electron transport from succinate to NAD resulting in the formation of NADH is higher when compared with that forming under direct oxidation of NAD-dependent substrates (456). The observed relationship between ROS formation and the redox state of the couple NADH/NAD resulted in the proposition that the ROS formation is directly proportional to the level of reduction of NAD. Possibly, a more generalized rule might be formulated that the more reduced the mitochondrial interior is, the more probable there will be primary ROS formation.

The redox state of the cellular milieu is mainly determined by the ratios of reduced/oxidized cofactors and proteins which carry the bulk of redox-sensitive amino acid residues and functional groups, NAD(P)H/NAD(P)$^+$ and GSH/GSSG, which all together form a compartmentalized redox buffer where all components are in a redox equilibrium under cellular steady-state conditions (reviewed in Ref. 309). This buffer may be an important factor in determining ROS levels in the compartments such as the mitochondrial matrix or cytosol (174). High intramitochondrial redox buffering capacity, only partially represented by 3–5 mM NAD(P)H and 2–14 mM GSH (416), would resist the short-term exposure to ROS, while profound sustained ROS exposure would eventually exhaust this buffer, resulting in the elevation of intramitochondrial ROS levels (353, 416, 460). Later, we discuss in greater detail the redox dependence of ROS formation and the role of reducing equivalents and mitochondrial membrane potential on the net ROS production (see sect. VB5).

The role of complex II in maintaining and modulating the mitochondrial/cellular redox environment remains undetermined. It is unknown whether in vivo mitochondria
reverse electron transfer from complex II to complex I occurs, and whether under physiological conditions the reverse electron transport could result in substantial ROS production considering that physiological concentrations of NADH would significantly attenuate $O_2^-$ production under conditions where reverse electron transport could be observed in in vitro model systems (165). Thus it remains questionable under normal conditions if there is a significant contribution of ROS generated in complex II to the net ROS production.

3. Under pathological conditions

As we discussed previously, the question about complex II contributing to the net ROS production remains controversial. Although the tissue level of succinate is as low as 200–500 μM, under oxygen deficiency (hypoxia/ischemia) it may rise 5- to 10-fold (44, 371, 471). Recently, significantly increased levels of succinate (to a few millimolar range) were detected in macrophages exposed to lipopolysaccharide, ultimately identifying succinate as a metabolite in innate immune signaling, which stabilizes HIF-1α and enhances interleukin-1β production during inflammation (433). Activation of macrophages is known to be associated with elevation of their ROS production, but whether succinate triggers this production remains unexplored.

Under some circumstances of drug-induced apoptosis when intracellular pH becomes significantly acidified, impairment of complex II could correlate with ROS generation without changes in the SDH enzymatic activity which is a part of complex II activity (260). This process has been accompanied by dissociation of the SDHA (flavoprotein subunit)/SDHB (iron-sulfur protein-containing part) subunits, which encompass the SDH activity, from the membrane-bound components of complex II that are required for the SQR activity (for details, see FIGURE 1). Such dissociation (see FIGURE 1) might result in a direct single-electron reduction of oxygen by a reduced iron-sulfur cluster of complex II (113). Consequently, it has been proposed that complex II may function as a general sensor for apoptosis (162, 260). This is an example of a pathological, conformation-induced ROS production which does not happen in intact complex II.

In a catalytic mechanism, ubiquinone receives electrons from the [3Fe-4S] center being bound at the Qp site. Since the ubiquinone is a two-electron acceptor receiving these electrons in separate steps, the intermediate state of SDH exists where after receiving a single electron the ubisemiquinone radical must be stabilized to prevent the escape of the electron to an inappropriate acceptor such as molecular oxygen. Mutations in the vicinity of the Qp site were shown (167) to compromise the ability to stabilize ubisemiquinone, and thus its unpaired electron may become more readily available to react with ambient oxygen producing its derivative, superoxide anion radical followed by dismutation to form hydrogen peroxide.

There is multiple evidence that impaired electron transport in SDH, as well as its effect on the levels of NAD(P)H through the impairment of the Krebs cycle, are the source and the cause of a substantial amount of ROS determining the onset of numerous pathologies (e.g., Refs. 32, 204, 481, 482). Malfunctioning of respiratory complexes, including complex II in brain mitochondria, is a hallmark of Huntington disease (HD), a neurodegenerative genetic disorder that elicits progressive motor, cognitive, and emotional deficits. 3-Nitropropionic acid, an irreversible inhibitor of SDH, mimics HD-like pathology and symptoms (68) and evokes an ROS increase in neurons (270). Leigh syndrome, an infantile-onset progressive neurodegenerative human disease is suggested to be caused by mutations in the SDHA gene. It appears that mutations in the SDHB, SDHC, or SDHD
genes can cause paragangioma (a neuroendocrine, highly vascularized neoplasm developing tumors in the head, neck, thorax, or abdomen) (37) or pheochromocytoma (a catecholamine-secreting neuroendocrine tumor occurring in the medulla of the adrenal glands) (37, 155; reviewed in Ref. 305). Unfortunately, it is impractical so far to estimate the contribution of the impaired Krebs cycle and reverse electron transport occurring under pathological SDH impairment to the modulating ROS production.

In parasitic worms residing in an anaerobic environment in a host intestine, the energy partially is obtained from so-called fumarate respiration reflecting a reverse activity of succinate-ubiquinone reductase of complex II. Apparently, in their fumarate reductase reaction, ROS are produced in a FAD site and quinone-binding site as well. Since in the adult stage, these worms do not have either complex III or IV, their respiratory chain could serve as a good model to study the production of ROS in complex II in mitochondria (364, 436). It is noteworthy that this model with a missing cytochrome c oxidase may somehow simulate either hypoxic conditions or those induced by defective electron transfer downstream of Complex II.

B. Complex I

1. Under normal conditions

The association of complex I deficiency with a wide spectrum of pathologies such as cardiomyopathies, cataracts, Leigh disease, exercise intolerance, mitochondrial encephalomyopathy, lactic acidosis, stroke-like episodes (MELAS), hepatopathy, and tubulopathy has been suggested. Prevaling dogma holds that complex I (NADH-ubiquinone oxidoreductase) is the main source of ROS in mitochondria. However, the ROS production at complex I depends on circumstances; consequently, complex I becomes a major ROS source under pathological conditions rather than being a dominant source under resting and healthy conditions.

When submitochondrial particles or isolated mitochondria oxidize NAD(P)H or glutamate plus malate, correspondingly, complex I production of superoxide is negligible. However, supplementation with the inhibitor of complex I, rotenone, results in robust production of $O_2^{-}$ (350, 456). This implies that the major site of ROS production in complex I is either upstream of a rotenone-binding site or it is tightly coupled to the increased level of NAD(P)H after rotenone supplementation during oxidation of NAD-dependent substrates (175, 444). According to the first alternative, rotenone would induce progressive reduction of the upstream redox groups (432) including Fe-S clusters, flavin mononucleotide (FMN), and the tightly bound pool of ubiquinone (62, 325), which can supply the oxygen molecule with a single electron yielding superoxide anion radical.

There is debate on the critical role of the components of complex I involved in superoxide production. Some consider FMN (247, 256, 287, 345, 455), while others claim that iron-sulfur clusters N1a and N2 (142, 152, 186, 254), NAD radical (245), or ubisemiquinone (241) are responsible for $O_2^{-}$ generation in complex I. The last point was actively challenged by Lenaz (264) who considered only hydrophilic quinones to be prooxidants, while physiologically hydrophobic ubiquinones (such as CoQ10) behave more as antioxidants rather than prooxidants (264). Therefore, the question regarding the major source of superoxide in complex I under physiological conditions remains unresolved.

Recently, the physiological relevance and thus the importance of the production of ROS by complex I was questioned on the basis that NADH-supported complex I-catalyzed superoxide production by submitochondrial particles shows maximal activity at low NADH concentrations (~50 μM) while at physiological concentrations of NADH (in the millimolar range) this reaction is severely inhibited (164, 165).

2. Under pathological conditions

It has been noticed that at least 40% of all mitochondrial disorders are associated with mutations in subunits of complex I (402). Defects in complex I are associated with a wide diversity of neurodegenerative pathologies, including Parkinson’s disease (PD) which is characterized by a substantial loss of the dopaminergic neurons and cell bodies of which are in the substantia nigra pars compacta and nerve terminals in the striatum. ROS are thought to be highly involved in PD pathogenesis, triggering the loss of redox buffers (GSH and proteinaceous thiols) (336) at least partly caused by dopamine oxidation-related metabolic pathways.

Dopamine in the central nervous system, apart from being a neuronal neurotransmitter, serves as a precursor of norepinephrine and epinephrine, and is a regulator of movement (nigrostriatal pathway), and a behavior motivator (mesolimbic pathway) (425). While under normal conditions oxidative deamination of dopamine by monoamine oxidase produces hydrogen peroxide (282), it could generate toxic oxidants through alternative ways of oxidation wherein mitochondria play a role. In this pathway, dopamine is oxidized nonenzymatically by superoxide forming dopamine quinone which can be reduced by mitochondrial complex I generating semiquinone followed by a transfer of its electron to molecular oxygen to form superoxide (488), completing a vicious oxidative cycle. In addition, PD is hallmarked by elevated iron levels that may catalyze production of deadly oxidants, possibly in a self-amplifying mode (411).

PD could be mimicked by the action of complex I inhibitors such as rotenone, pararquat, and 1-methyl-4-phenyl-1,2,3...
3,6-tetrahydropyridine (49, 94). Exposure to the latter drug has shown to produce permanent parkinsonism in humans, non-human primates, and rodents, by exerting an effect primarily on the function of mitochondrial complex I. In patients with Friedreich ataxia, a deficient activity of the Fe-S cluster-containing subunits of mitochondrial respiratory complexes I, II, III, and aconitate was found (361). Kushnareva et al. (254) claimed that the ratio of NAD(P)H/NAD(P)+, rather than the level of NADH, determines reduction of ROS-producing sites in complex I.

Generation of ROS associated with hypoxia/reoxygenation is known as one of the most deleterious causes of oxidative damage. Three potential sources of ROS have been proposed to be responsible for this release: mitochondrial complex I, xanthine oxidase, and NADPH oxidase (2, 471, 491). However, the latter two are probably not involved since inhibition of these complexes in vivo did not afford cell protection (84, 129).

One of the very specific features of the mammalian NADH-ubiquinone oxidoreductase is the slow active/deactive state transition, suggesting gross conformational rearrangements of complex I, at least in that part which is involved in rotenone-sensitive ubiquinone reduction [which may be involved in the superoxide production (150, 454)]. It was found that complex I isolated from the heart which was exposed to a normoxic perfusion is in a fully active state, while 30-min anoxic perfusion results in a significant transformation of the enzyme into a deactive state which returns back to normal after reoxygenation (283). It has been proposed that these conformational transitions can be relevant to producing ROS by complex I after cardiac tissue is re-oxygenated following a coronary occlusion (283). Using EPR spectroscopy, DeJong et al. (104) showed that NADH-coenzyme Q oxidoreductase undergoes energy-dependent structural changes in parts determining ubisemiquinone production (iron-sulfur cluster 2) (104). Thus, under pathological conditions, conformational rearrangements may be involved in the changes of the efficiency of ROS-producing machinery in complex I.

3. ROS and hypoxia

The reaction of formation of a primary ROS (superoxide only) generated in the respiratory chain from molecular oxygen is of a first order with respect to oxygen concentration. However, paradoxically, generating ROS in mitochondria in the cell remains constant or even increases when PO$_2$ drops dramatically (i.e., under moderate hypoxic conditions). Robust ROS production under 1.5% of O$_2$ has been recently recorded (314, 374, 462).

Interestingly, in the cell the affinity of molecular oxygen to ROS-generating modules is higher than to cytochrome oxidase. This obviously takes place under conditions of partial reduction of cytochrome oxidase, i.e., when the availability of oxygen is limiting its utilization. Note that this paradox is absent in the isolated mitochondrial system (FIGURE 2), free from extramitochondrial signaling pathways which confirms that elevated mitochondrial ROS generation in the cell in response to hypoxia is not intrinsic to the mitochondrial respiratory chain alone but can be attributed to some involvement of extramitochondrial factors (189). Marshall et al. (286) indicated that hypoxia-induced superoxide production occurs through activation of NADPH oxidase located in the cell membrane. In addition, under moderate hypoxia, NO synthesis in mitochondria continues although being only 5–10% of the normal steady-state level (K$_m$ for oxygen of the mitochondrial NO synthase is 30–40 μM; Ref. 8). In turn, NO can partially block cytochrome oxidase (69, 70, 378), thus reducing mitochondrial electron carriers, increasing its K$_m$ for oxygen (91) and favoring generation of superoxide at hypoxic conditions (444).

In highly metabolizing tissues, the areas surrounding mitochondria in the cell may have higher ROS levels than remote areas. Without considerable mitochondrial ROS-quenching activities, intramitochondrial levels of ROS may potentially reach very high levels. That may happen in case of an imbalance between the oxygen supply and demand, for example, under conditions of high metabolic needs.

The nature of the tissue oxygen gradient between the source of oxygen (blood capillary) and the site of its utilization (the mitochondrion) can be explained by the Krogh cylinder model which generally serves to analyze capillary tissue exchange kinetics (246, 266) (FIGURE 3). The effective radius of this cylinder beyond which the tissue hypothetically is experiencing hypoxia depends in part on O$_2$ consumption. In a tissue with high metabolic rate (such as heart muscle), capillary density during maximum or moderate exercise would not be sufficient to supply tissue with oxygen and might potentially produce more ROS in the vicinity...
and inside of mitochondria/mitochondrial clusters (214). However, basic theoretical assumptions of the Krogh cylinder model do not consider that the diffusion coefficient for O$_2$ in muscle tissue may be higher due to the possibility of facilitated O$_2$ transport by the mitochondrial network (10, 396) and/or by myoglobin molecules (475). In non-muscle tissues lacking myoglobin, the cytoglobins and neoglo-

4. Is the ROS production proton motive force sensitive?

Proton motive force (pmf, $\Delta$\textit{p}) across the inner mitochondrial membrane, with $\Delta$$\psi$ as a main component, is driving ATP production (268, 298, 299, 424). Whether ROS production is dependent on this proton motive force/trans-

Redox steady states of respiratory components responsible for ROS production should be in redox equilibrium with adjacent mobile and immobile redox carriers such as NAD(P)H, GSH, and the thiol groups of the proteins occupying the same compartment.

Under experimental conditions acutely restricting the oxygen supply to an organ, such as the heart, very steep regional redox transitions have been observed across the borderline of the ischemic area (33), apparently reflecting a similarly steep oxygen gradient and thus probably the ROS gradient as well (FIGURE 4). On the basis of the data that ROS production is directly linked to reduced equivalents such as NADH (discussed above), hypoxic regions manifesting the highest level of NAD reduction would likely achieve much higher ROS levels than normoxic regions. The brain is the organ most vulnerable to the lack of oxygen, immediately responding by the reduction of NAD (78), thus potentially eliciting ROS within local areas adjacent to ischemic zones.

As discussed above, a reduced redox intramitochondrial environment is a prerequisite for high primary ROS forma-
As was shown in the preceding section (FIGURE 5), moderate lowering of Δψ could result in a lower ROS production in mitochondria without a significant effect on ATP production. This could be achieved by inducing a small proton leak through the inner mitochondrial membrane which would both stimulate oxygen consumption and, in parallel, shift the level of reduction of mitochondrial ROS-producing sites to a more oxidized state lowering the probability of ROS production in the mitochondria. While higher potential could drive increased ATP production, the higher Δψ could also result in the production of increased ROS levels and potentially unwanted oxidative consequences. Therefore, achieving a reasonable balance between ROS and ATP production by mitochondria is crucial since this reflects the current energy needs of the cell under the particular physiological state. As discussed previously, regulated moderate (“mild”) uncoupling of mitochondrial oxidative phosphorylation has been suggested as a feasible therapeutic strategy (397, 398, 415) for regulation of the intracellular and intramitochondrial ROS level (99).

Mitochondrial uncoupling proteins (UCPs) have been considered as potential mild uncouplers. The relationship between ROS production and UCPs activity was revealed in 1997 in experiments where GDP, an inhibitor of UCP1, caused an increase of Δψ and ROS production (316). Later, it was demonstrated that superoxide directly activates UCPs resulting in a negative feedback controlling both ROS production and their levels (120).

Mild uncoupling may be protective against excitotoxic injury (469) and against injury of dopaminergic neurons in substantia nigra from mitochondrial poisons such as rotenone (478). Decreasing ROS generation by uncoupling mitochondria increases longevity in healthy animals (74).

Of all the possible mild uncouplers, fatty acids are probably the most natural ones (240, 398, 476). In their protonated form they can cross the mitochondrial inner membrane followed by deprotonation in the matrix side, and then the anionic form of the fatty acid completes the cycle by returning back to the cytosolic side. The rate-limiting step of this cycle is the transport of anionic form. Different proteins such as the adenine nucleotide transporter (ANT) and glutamate/aspartate transporter are involved in fatty acid-mediated uncoupling through facilitation of the transport of the anionic form (12, 368). Thyroid hormones may also be considered as natural mild uncouplers (177, 397).
Among artificial uncouplers, 2,4-dinitrophenol (DNP) has been tested as an anti-obesity drug, but it was found to be too toxic for practical use (90, 99). Additionally, DNP was found to limit the experimental infarct size in the heart and brain, and this was interpreted to occur through diminishing the ROS level (238, 359). Recently, mild uncoupling activity was ascribed to a series of derivatives of cationic rhodamine (15).

C. Complex III

Complex III (ubiquinol-cytochrome c oxidoreductase) accepts reducing equivalents formed in complexes I and II and processes them by the Q-cycle operating mechanism. Operation of this cycle is initialized by ubiquinol, which releases its proton to the intermembrane space and donates one electron to the Riske iron-sulfur protein (which can bind to, and be inhibited by, myxothiazol) producing unstable semiquinone on the outer side of the inner mitochondrial membrane. The semiquinone serves as an electron donor for hemes of cytochrome $b_{1}$, and then of cytochrome $b_{11}$ which is located close to the inner side of the membrane. Cytochrome $b_{11}$ reduces ubiquinone in an antimycin A-sensitive way producing ubisemiquinone followed by its further reduction with a second electron and protonation (442) (FIGURE 6).

Under normal conditions, the probability of existence of unstable semiquinone ($Q^{-}$) is low due to its fast oxidation; therefore, the probability of donation of one electron to molecular oxygen in this system is relatively low. Only the block of the electron flow by antimycin A results in a high superoxide release apparently due to the reduction of both hemes of the cytochrome $c$ in parallel with the elevation of the steady-state level of semiquinone, thus giving a higher chance of one-electron reduction of oxygen (FIGURE 6). Among all of the mechanisms presented in the scheme, inhibitors of bc$_{1}$ complex (antimycin A, myxothiazol, and stigmatellin), antimycin is the only effective ROS inducer, although some low level of superoxide production could be detected in the presence of other inhibitors (418) in the site of bc$_{1}$ complex different from that induced by antimycin A. Therefore, the potential role of complex III as a cause of gross mitochondrial ROS production under the physiological steady-state mode of operation remains uncertain considering that substantial mitochondrial ROS release occurs only after application of a drug having no natural analogs in animal physiology. Noteworthy, as in the case of ROS production in complex I, conformational changes detected in bc$_{1}$ complex after antimycin A binding (45, 95, 193, 355) may be a prerequisite for dramatic molecular rearrangements in the complex resulting in a marked amount of ROS production.

Overproduction of ROS by complex III may result from acquired and genetic defects in the mitochondrial respiratory chain in close proximity to an antimycin A-binding site. B. Chance’s lab performed a study on a patient who was diagnosed with a deficiency of cytochrome $b$ in complex III, resulting in muscle weakness associated with a ragged-red fiber myopathy and lactic acidosis (122). The total succinate-cytochrome $c$ reductase activity in skeletal muscle of this patient was only ~5% of normal. It was already known that menadione can shuttle at least a portion of electrons over an antimycin-sensitive site (319). The treatment of this patient with menadione bridging electrons from coenzyme Q directly to cytochrome $c$, thus bypassing the defective cytochrome $b$, resulted in a significant therapeutic effect with partially normalized muscle exercise tests. This approach, named “redox therapy,” demonstrates the importance of de-
tailed understanding of mitochondrial redox-related pathogenesis.

D. Importance of Redox State of NAD(P)H/ NAD(P)⁺ in Mitochondrial ROS Production

A comprehensive analysis of the relevance of NADH in managing mitochondrial production of ROS has been performed by Vinogradov’s group (163, 165). With the use of submitochondrial particles oxidizing NADH, it has been found that a substantial amount of superoxide production took place only when 50 μM NADH was used, while in the presence of 1 mM NADH, the production was remarkably suppressed. NAD⁺ revealed the same superoxide suppressive ability (165). Considering that physiological concentration of the couple NAD⁺/NADH in the mitochondrial matrix is in range of a few millimolar (474), with a significant fraction of it existing in a free form, the gross generation of ROS mediated by complex I may be almost negligible. Similar experiments with isolated permeabilized mitochondria and the soluble protein fraction of the mitochondrial matrix showed the same result. The authors concluded that complex I was not a primary source of ROS in mitochondria under physiological conditions. Instead, they hypothesized that some oxidoreductases poised in equilibrium with NAD(P)/NAD(P)H may be that primary mitochondrial source of ROS (165).

In isolated permeabilized mitochondria, the same authors detected quite high NADH-dependent H₂O₂ production when they supplemented the system with ammonium salts (163). The mitochondrial H₂O₂ release was insensitive to dicumarol (inhibitor of NADH-quinone oxidoreductase, D,T diaphorase) and NAD-OH (inhibitor of complex I), suggesting the matrix localization of H₂O₂-producing activity. This ROS-generating activity depended on the ratio of NAD(P)⁺/NAD(P)H. It was concluded that a specific ammonium-sensitive NADH oxidase activity in the mitochondrial matrix is responsible for this H₂O₂ production, but the in vivo relevance of this process is still unknown. [An alternative explanation for an ammonium effect could be the hypothesis that mitochondrial matrix alkalinization (caused by ammonium entry) increases superoxide production by stabilizing semiquinone radical (381).]

Further analysis of the nature of this ammonium-stimulated enzyme producing primary mitochondrial ROS revealed that the questionned enzyme possessed NADH:lipooamide oxidoreductase activity and later was identified as dihydro- lipoyl dehydrogenase (224). Dihydrolipoyl dehydrogenase is an essential component (called E3 component) of two mitochondrial redox complexes: α-ketoglutarate dehydrogenase complex (KGDC) and pyruvate dehydrogenase complex (PDHC). This mitochondrial enzyme contains FAD [which contributes mostly to the overall mitochondrial autofluorescence signal originating from cellular flavins (178, 250)] with the redox state in equilibrium with the environmental NAD(P)H/NAD(P)⁺. It has been found that E3 is responsible for superoxide and hydrogen peroxide generation in purified KGDC and PDHC as well as in KGDC operating in mitochondria in vitro (71, 151, 417, 420, 438).

It is not surprising that ammonium has an effect on the component of α-KGDHC since α-ketoglutarate, instead of converting into succinyl CoA in the citric acid cycle (the Krebs cycle), can be transformed into glutamate by glutamate dehydrogenase. Typically, this reaction does not take place in mammals, since the equilibrium of the reaction is shifted toward the reverse direction, but it may occur in toxic levels of ammonia. Ammonia metabolism is important in all tissues. However, in the brain for which a high level of ammonium is extremely toxic (59), it becomes a critical element involved not only in the detoxification process (by astrocytic glutamine synthase and the all-mitochondria-located urea cycle) but also in a number of essential biochemical reactions in the cell as part of the brain signaling modules (i.e., glutaminase reaction to maintain optimal cycling of glutamine/glutamate; Refs. 421, 422, 448). With the consideration of results mentioned above, the ammonium toxicity might be at least partially mediated by the mitochondria-formed ROS.

E. Other Mitochondrial ROS-Producing Sites

1. NADPH-oxidase

The prototypic NADPH-oxidase (Nox) has been found in the plasma membrane of phagocytes and B lymphocytes, and it is involved in the phagocytic activity by ejecting superoxide radical which is a primary element igniting antibacterial defense. Its membrane domain is represented by a protein gp91PHOX (PHOX for phagocyte oxidase) which can be organized as a heterodimer in combination with other cytosolic proteins from the PHOS family which all together form flavocytochrome b₅₅₈ complex (6, 24).

So far, seven isoforms of Nox (Nox1–5, Duox1–2) have been identified, with all having distinct catalytic domains. The above-mentioned form is called Nox2. It has been found not only in the cell membrane, but also in the cell interior. Other isoforms reside in different specialized tissues and different intracellular loci. Nox4 is the only member of the family found to have a mitochondrial localization [in cultured mesangial cells (55), rat kidney cortex (55) and cardiac myocytes (251)]. Nox4 differs from the other members of the Nox family in that it preferentially produces H₂O₂ rather than O₂⁻ (356). The discovery of its mitochondrial localization conflicts with data that shows that the natural cytosolic partner of Nox4 complex, p22PHOX, whose presence affords NADPH oxidation
was not found in mitochondria, and furthermore, that the specific activity of Nox in mitochondria is not measurable (111). Considering the high importance of NA-DPH-oxidase, specifically Nox4, in angiogenesis (483) and pathogenesis of atherosclerosis, diabetic injury (279) and other pathologies (280) including aging (4), further experimental research is needed to resolve this apparent conflict and gain a better understanding of the relevant aspects of Nox-mediated redox signaling.

An interesting mode of interaction between Nox and mitochondria was shown recently in cancer cells where glucose deprivation provoked a signaling-based positive-feedback loop that amplifies ROS levels above a toxicity threshold resulting in cell death (159). This positive-feedback loop involved the complex integration of homeostatic control mechanisms for metabolism (particularly, redox balance established by Nox and mitochondria) and tyrosine kinase signaling through regulation of protein tyrosine phosphatases. According to the authors, glucose withdrawal activates supraphysiological phosphotyrosine signaling and ROS-mediated cell death. In cancer cells that are highly dependent on glucose for survival, glucose and pyruvate deprivation induces oxidative stress driven by Nox and mitochondria. This oxidative stress provokes a positive-feedback loop in which Nox and mitochondria generate ROS and inhibit tyrosine phosphatases by oxidation. With the negative regulators turned off, tyrosine kinase activates Nox, further amplifying ROS generation and provoking cell death.

2. Monoaminoxidase

Monoaminoxidase (MAO) resides in the outer mitochondrial membrane and serves as a marker there. This flavoenzyme has two isoforms A and B with different substrate specificity and sensitivity to inhibitors (121). Their substrates are biogenic amines whose oxidation yields in the generation of corresponding aldehydes, H$_2$O$_2$, and ammonium base. MAO activity has special importance in the brain where peroxidase and catalase activities are low to fully decompose H$_2$O$_2$ formed during oxidative deamination of neurotransmitters (e.g., dopamine, serotonin), thus significantly depleting the endogenous pool of reduced glutathione (370).

The H$_2$O$_2$-generating activity of MAO might be the highest among all mitochondrial ROS generators. Isolated rat brain mitochondria produce H$_2$O$_2$ during oxidation of the exogenous amine, tyramine (at supraphysiological 2 mM concentration) at a rate 45.2 μM/s (179), while H$_2$O$_2$ production during succinate oxidation in the presence of antimycin (considered to be the “gold standard” method for mitochondrial ROS production) is 0.95 μM/s (179, 334), i.e., MAO activity is 48 times more H$_2$O$_2$-generating than complex III. Oxidation of tyramine by brain mitochondria results in oxidative damage of mitochondrial DNA which is abolished by MAO inhibitors (179). Compared with normal conditions, MAO produces much more H$_2$O$_2$ during ischemia/reperfusion of the brain (389), the kidney (249), and the heart (50, 221). MAO activity in cardiac mitochondria of 24-mo-old rats was about eight times higher than that in 1-mo-old rats, demonstrating that MAO may be an important source of ROS in the aging heart (109, 288). However, the basic MAO activity in normal tissue is quite low due to the limitation of the availability of endogenous substrates of oxidation (such as serotonin, epinephrine, norepinephrine, dopamine, and others present in the brain in only nanomolar concentrations). On the other hand, chemical inhibitors of MAO elevate ROS production in cells (50–52). Paradoxically, the ablation of MAO causes a very slight rise of its endogenous substrates in the brain tissue (386). Because the levels remain in the nanomolar range, it is doubtful that these substrates could contribute significantly to overall ROS production in tissue. However, close proximity to the sites where biologically active amines are formed and released (such as synapses and extraneuronal compartments such as astrocytes and glial cells) and the high mitochondrial density in and around these areas, may render mitochondrial MAO an important component in both inactivation of amines and the local rather than overall ROS production in such areas. It has been speculated that under normal physiological activity, ROS produced by MAO in these areas performs metabolic and signaling functions in the brain (31). In addition to ROS, the by-products of biologically active amine conversion by MAO may play a direct role in degenerative processes, e.g., the dopamine molecule after entering MAO reaction produces a reactive quinone that could modify and damage cellular components (425).

3. p66$^{shc}$

On the basis of the observation that p66$^{shc}$-deficient mice display extended life span, remarkably reduced levels of ROS, and increased tolerance to oxidative stress, it has been suggested that p66$^{shc}$ could have an important role in ROS production and aging (295, 328, 439). Normally p66$^{shc}$ resides in the cytosol, while under oxidative stress (e.g., under ischemia/reperfusion insult), it could be translocated in the mitochondria in a PKC$\beta$-dependent way (341) where it serves as an important source of ROS (109). In mitochondria, this adapter molecule has been suggested to function as a redox enzyme possibly oxidizing cytochrome $c$ and generating H$_2$O$_2$ in the amino-terminal portion of p66$^{shc}$ containing sequence similar to that of certain redox enzymes (156).

It was hypothesized that p66$^{shc}$ in mitochondria exists within a high-molecular-weight complex which includes mtHSP70 and TIM-TOM complex. Inside of such a complex, p66$^{shc}$ is inactive. After propagation of apoptotic signal, the complex is dissociated resulting in the release of free p66$^{shc}$ which becomes activated and capable of participat-
ing in the electron transport which generates H_2O_2. Mutations in the redox active sequence of p66^{shc} abolish its pro-apoptotic activity apparently through the inability to interact with cytochrome c (156). Recent data show that p66^{shc}-generated ROS regulate insulin signaling (48), T-cell and B-cell signaling pathways (133), and expression and activity of the ROS-generating enzyme NADPH oxidase (NOX4) as well as activate NF-κB (226, 291), thereby amplifying oxidative stress and inflammation.

4. α-Glycerophosphate dehydrogenase

Another potential source of ROS in mitochondria could be α-glycerophosphate dehydrogenase that occupies the outer surface of the inner mitochondrial membrane, but its activity is relatively low in the liver, heart, and brain but high in brown adipose tissue (304). However, isolated mitochondria supplemented with α-glycerophosphate in the presence of antimycin A produce hydrogen peroxide which, when normalized to an enzymatic activity, exceeds that originating from complexes I or II (116). This source could represent one of the most efficient ROS generators in mitochondria. It is almost insensitive to the presence of an uncoupler or rotenone, implying that ROS generation by α-glycerophosphate oxidation is not dependent on mitochondrial Δψ contrary to, e.g., succinate oxidation (304). The detailed mechanism of ROS production by this enzyme is not defined yet.

In addition to previously mentioned sources of mitochondrial ROS production, it was shown that cytochrome b_3 reductase (470) and dihydroorotate dehydrogenase (112, 140, 276) produce ROS on the outer surface of the inner membrane. However, the significance of these enzymes in the total ROS production remains questionable.

5. Electron transfer flavoprotein (ETF) and ETF quinone oxidoreductase (ETF dehydrogenase)

In 1972 Boveris et al. (58) found that rat liver mitochondria can produce a substantial amount of hydrogen peroxide when oxidizing palmitoyl carnitine or octanoate. This production was ceased in the presence of an uncoupler. The fatty acid-induced ROS generation was comparable to that in the presence of glutamate plus malate and was slightly lower with succinate (58). Thirty years later, similar results were obtained with skeletal muscle and heart mitochondria (414). Increased lipid metabolism was correlated with upregulated UCPs expression/activities (73, 369), possibly to salvage the system from excessive ROS production (414). Since ROS generation was almost insensitive to external SOD, it has been proposed that the fatty acid β-oxidation may result in ROS generation at a distinct mitochondrial matrix site which is different from o-center of complex III. In addition, it has been proposed that a significant contribution to ROS production during fatty acid oxidation comes from ETF which accepts electrons from different dehydrogenases including those involved in β-oxidation (41) and transfers them to the ubiquinone pool in the inner mitochondrial membrane by a reaction catalyzed by ETF-ubiquinone oxidoreductase (ETF-QO) residing in the matrix side of the inner mitochondrial membrane (153). In β-oxidation, the sequence of reactions is as follows: acyl CoA dehydrogenase → ETF → ETF-QO → Ubiquinone → complex III.

ETF-QO contains flavin (FAD) and a [4Fe-4S] cluster which makes it vulnerable to ROS (363), and it serves as a convergence point for electrons flowing from nine flavoprotein acyl-CoA dehydrogenases and two N-methyl dehydrogenases (153, 227). Recently, it was found that oxidation in muscle mitochondria of long-chain fatty acids in physiological (low) concentration is associated with higher rates of ROS formation than oxidation of NADH-linked substrates while exhibiting relatively low dependence on the mitochondrial membrane potential (380). The authors suggest that this enzymatic activity may be responsible for Δψ-independent ROS production.

Deficiency of ETF-QO in most cases is caused by single point mutations around the FAD-ubiquinone interface (326) and results in a human genetic disorder known as multiple acyl-CoA dehydrogenase deficiency (MADD) or glutaric acidemia type II (141). This is characterized by impaired fat and protein metabolism. It may be associated with acidosis or hypoglycemia and accompanied by other symptoms such as general weakness, liver enlargement, increased risk of heart failure, and carnitine deficiency, and could result in a fatal metabolic crisis (144, 390, 443).

6. Aconitase

Aconitase catalyzes transformation of citrate to isocitrate in the Kreb cycle. It contains a cubane-type [4Fe-4S] center with three iron atoms interacting with cysteine residues and inorganic sulfur atoms, while the fourth iron, Fe-α, is exposed to the solvent that allows the catalytic dehydration of citrate to form the intermediate cis-aconitate, as well as the subsequent hydration of cis-aconitate to form isocitrate (42, 138). The prosthetic group of aconitase is highly susceptible to inactivation by superoxide anion radical yielding an inactive [3Fe-4S] form, Fe^{2+}-α and H_2O (138, 267, 452). Interaction of the latter two ignites Fenton’s reaction resulting in the release of OH radical (452). It has been proposed that aconitase would be an ideal sensor for ROS in cells (147, 148). Subsequently, superoxide toxicity in mitochondria could be explained by enhanced aconitase inactivation and related processes. For example, doxorubicin cardiotoxicity was explained mainly by an aconitase inactivation accompanied by hydroxyl radical release (296). Aconitase inactivation is an example of a “ROS cross-talk” where one type of ROS (superoxide) is inducing the release of another, potentially more damaging, ROS.
(the hydroxyl radical). An alternative point of view is that aconitase inactivation may serve a protective role by diminishing electron flow through the ROS-generating respiratory chain. In addition, the accumulation of citrate as a result of a decreased Krebs cycle flux promotes chelation of Fe\(^{2+}\) which is then irreversibly oxidized to a more stable complex citrate-Fe\(^{3+}\), thus preventing catalysis of Fenton’s reaction by free Fe\(^{3+}\) (400).

VI. ROS-INDUCED ROS RELEASE ASSOCIATED WITH THE mPTP

A. Fundamentals of the Discovery

The importance of cellular redox homeostasis in progression of inherited and acquired pathologies, including those associated with an aggressive oxidative environment, was postulated elsewhere (reviewed in Refs. 72, 174). As we discussed earlier, the redox homeostasis is determined by the balance between ROS generation matching metabolic needs and ROS quenching capacity. Undoubtedly, tipping the balance in favor of increased ROS production within the cellular microenvironment can severely alter the cellular redox equilibrium, potentially resulting in oxidative stress which when mild can cause oxidation of essential mitochondrial components. In extreme cases it can irreversibly damage these components resulting in a cell death. Within the great diversity of types of cell death, which to date comprise as much as 13 types (145), at least some of them could be associated with induction of the mPTP.

mPTP opening is a phenomenon known in the field of mitochondrial research for many decades, but for the first time described in details in a set of three consecutive papers by Haworth and Hunter in 1979 (182, 195, 196). This phenomenon [also recognized as an opening of a megachannel (228, 229, 430)], originally studied in isolated mitochondria, represents a sudden change in the permeability of the inner mitochondrial membrane allowing not only protons but also other ions and solutes of a size up to \(~1.5\) kDa to go through this membrane. There are many reviews on the tentative nature and identity of the mPTP (64, 489, 494) with details which are far beyond the scope of the present review. Previously, many candidates were considered to serve as the core of the pore [i.e., mitochondrial VDAC, cyclophilin D (cyPD), ANT (108, 170, 217, 351, 457, 489, 494)], but largely they have been all dismissed because of various reasons, but still leaving for them important pore-modulating functions. Recent evidence suggests that a dimer of mitochondrial ATP-synthase is essential to form a core of the mitochondrial pore (157) and \(c\) subunit of the mitochondrial ATP synthase complex may be required for mPTP-dependent mitochondrial fragmentation and cell death (56).

Although mPTP induction is typically referred to as a pathological event very often resulting in the degradation of mitochondria or the cell (which will be discussed later), there is multiple evidence and assumptions that in fact it can also serve physiological functions. It has been postulated that mPTP could serve as a release valve for quick release of cations constantly leaking into the mitochondrial matrix due to the mitochondrial membrane potential. A flickering mode of mPTP may serve this purpose with the mPTP opening for a time not sufficient for the onset of complete mitochondrial depolarization (239). Another support of a physiological role of the mPTP was obtained in CyPD knockout mice which demonstrated an obvious maladaptive phenotype in their hearts (124). While the role of CyPD as a core of the mPTP has been dismissed, there is a general consensus that CyPD can serve a modulatory role in the process of the mPTP induction. It was shown that CyPD knockout mice exhibit substantially greater cardiac hypertrophy, fibrosis, and reduction in myocardial function in response to pressure overload stimulation than control mice while cardiomyocyte-specific transgene expression of CyPD in these mice helped to rescue from the named pathologies. Also, in mice lacking CyPD ischemic preconditioning was augmented (239) while mPTP openings in wild-type mitochondria were much more frequent than in mitochondria of knockout mice. This supports the notion that the mPTP might have an important physiological role, possibly through regulation of intramitochondrial Ca\(^{2+}\).

The role of Ca\(^{2+}\) in the induction of the mPTP has been already mentioned above; similarly, the role of oxidants in generation of the mPTP pore is essential too, although both resulting in the same phenomenological outcome (255, 277). It has been recognized that the mPTP induction represents a highly complex phenomenon (322, 409). Particularly, isolated mitochondria exposed to Ca\(^{2+}\) plus P, demonstrate the collapse of \(\Delta\psi\) preceding mitochondrial swelling, suggesting that the generation of a smaller, low-conductance pore occurs with permeability for ions but not for solutes prior to induction of the mPTP (full size) pore (5, 67, 244, 339). Oxidants such as hydrogen peroxides, organic peroxides, and some other inducers generate both pores, but the insensitivity of induction of low-conductance pore to Ca\(^{2+}\) and insensitivity of fully induced mPTP to conventional inhibitor, cyclosporine A and sometimes to EGTA (65, 243, 262, 284), makes them different from classical pore inducers (255; reviewed in Ref. 489). Therefore, the mitochondrial pore phenomenon appears to be multi-faceted.

Light is known to be a potent oxidant inducer when it interacts with photosensitizing agents, and such a property is successfully utilized in photodynamic therapy (114, 317). It is based on the ability of the excited fluorophore to generate primary ROS which after release inside of the biolog-
ical sample may become destructive for cellular components. In principle, many biological molecules and cell compartments can be fluorescently labeled and oxidatively modified after interaction with excitation light. The mitochondrial Δψ provides selectivity for photodynamic action localized exclusively in mitochondria without potential impact on other intracellular compartments (301, 303, 366, 367, 403). After exposure to light, the photosensitizer, e.g., tetramethyl rhodamine methyl ester (TMRM) which is a conventional probe for mitochondrial membrane potential widely used for visualization of energized mitochondria in the cell, generates various ROS in water including very strong oxidants such as superoxide anion radical and hydrogen anion radical (491).

Because mitochondria are critical intracellular loci of ROS production, together with the fact that ROS exposure can lead to the mPTP, it was hypothesized that under certain circumstances the mPTP could become self-amplifying and unstable (491). This hypothesis was tested in cardiac myocytes, taking advantage of the unique organization of mitochondria between myofilaments in an ordered three-dimensional latticelike array forming straight lines thus allowing specific applications of line-scan confocal microscopy to address this question (491, 493). For this purpose, rat cardiac myocytes are double stained with the probe for mitochondrial membrane potential (TMRM or ethyl derivative, TMRE having excitation maximum in a green region of the spectrum) and the ROS probe, 2,7-dichlorodihydofluorescein diacetate (DCF-H₂). DCF-H₂ itself is nonfluorescing and unreactive toward oxidants; however, after base hydrolysis of the ester bonds, the resulting nonfluorescing compound becomes reactive toward ROS, and after oxidation it is transformed into fluorescing DCF with maximum excitation in the blue region of the spectrum. The first scan of a cardiac myocyte previously unexposed to light frequently reveals the area(s) in which mitochondria are expected to occupy, but unstained with TMRM (suggesting that mitochondrial Δψ had collapsed in these mitochondria) but also demonstrating high DCF fluorescence. The size of the area is dependent on the physiological status of the cell (FIGURE 7), possibly reflecting the cellular level of tolerance to ROS. Therefore, isolated adult rat cardiac myocytes are a convenient model to study cellular oxidative stress.

B. mPTP and Ischemia/Reperfusion Injury

For the last 50 years it has been well recognized that coronary reperfusion of infarcted myocardium is associated with increased necrotic death of irreversibly injured cardiac myocytes. Jennings et al. (208) were first to report harmful, both structural and functional, changes associated with reperfusion. Therefore, early reperfusion while crucial for preserving ventricular function, preventing infarct expansion and potential development of heart failure, may also contribute to the pathogenesis of reperfusion arrhythmias and myocardial stunning manifested by reversible contractile dysfunction. The biological basis of reperfusion injury has been extensively studied since then and consequently the notion of reperfusion as a double-edged sword was formulated in 1985 by Braunwald and Kloner (63). In 1986 ischemic preconditioning was described as a means to render the heart more resistant to ischemia/reperfusion injury (311). The importance and the clinical potential of these discoveries has prompted the research community to focus on deciphering the molecular mechanisms that underlie rep-
Mitochondria have been implicated as a potential source as well as a target of the generated ROS resulting in an observed loss in mitochondrial function during ischemia/reperfusion and consequent irreversible cellular injury (9, 101, 329). It has been noted that not only these radicals play a significant role in the tissue damage observed following ischemia/reperfusion but that this injury can be mitigated by oxygen radical scavengers (e.g., Ref. 213). In the early 1990s, Crompton and colleagues demonstrated in isolated mitochondria that the derangement of mitochondrial bioenergetics that develop on reoxygenation, when resting cytosolic Ca²⁺ is high and ATP low, could lead to excessive mitochondrial Ca²⁺ uptake and consequent induction of mPTP (96, 97). This results in mitochondrial uncoupling and activation of ATP hydrolysis by F₁F₀-ATP-synthase (96, 97, 118).

The cardiomyocytes exposed to the hypoxia-reoxygenation cycle have much larger areas occupied with fully deenergized mitochondria and high levels of ROS, and these levels were diminished and Δψ was regained in most mitochondria after ischemic or pharmacological preconditioning of the cell (FIGURE 7) (218). Based on the previously expressed assumption that the cardiac ischemia-reperfusion injury is associated with the induction of the mPTP (97, 161), the speculation was put forth that the found abnormal subcellular loci in cardiac myocytes were indeed occupied by mitochondria which had undergone permeability transition. The most striking detail was that mitochondrial deenergization, if caused by the mPTP opening, was associated with higher, rather than lower, ROS production as follows from FIGURE 5.

C. RIRR: Experimental Demonstration

To further explore this puzzling phenomenon, confocal microscopy was employed in line-scan mode allowing repeated excitation of a row of mitochondria along the selected line in cardiac myocyte (FIGURE 8, A–C). Within the light-exposed mitochondria, a sudden loss of Δψ with corresponding rise of ROS generation was revealed, suggesting mPTP induction. Although these transitions were modestly sensitive to cyclosporine A, they were dramatically delayed by another mPTP inhibitor, bongkrekic acid, and by a ROS scavenger, Trolox. Furthermore, re-
concluded that the collapse of the mitochondrial mPTP induction also confirmed opening of a pore permeable to a 620-Da compound (FIGURE 8B). Thus it was concluded that the collapse of the mitochondrial ΔΨ occurs due to the mPTP pore opening.

Fine analysis of the ROS burst kinetics associated with the mPTP induction showed that ROS generation in the affected mitochondrion proceeds in two distinct phases: the initial, slow rise due to the accumulation of a photochemistry-associated production of ROS (called “trigger ROS”) and the subsequent ROS burst associated with dissipation of the mitochondrial membrane potential. This biphasic process was named “ROS-induced ROS release” (abbreviated RIRR). In addition to the ROS burst and the mPTP induction, it was accompanied by the burst of nitric oxide production; thus the term ROS-induced ROS/RNS release where RNS stands for reactive nitrogen species is also relevant (491).

The identical phenomenon using the same instrumentation as in original RIRR study (491) was later described in cells infected by adenovirus carrying mitochondria targeted circularly permuted yellow fluorescent protein (cpYFP) as the ROS sensor. The term superoxide flashes was used to describe the RIRR in transfected cardiac myocytes (461). Recently, this approach was reexamined and challenged by observation that the cpYFP is very sensitive to pH changes, therefore rendering this interpretation inconclusive (376, 377, 466).

The complex I inhibitor rotenone (at low concentrations not affecting TMRM sequestration) significantly decreased the ROS burst magnitude, confirming the mitochondrial nature of the ROS burst during mPTP-associated RIRR (491). The few second delay of NADH oxidation following the collapse of the mitochondrial ΔΨ suggests that NADH is the redox-energy store driving the electron donor necessary to support the single-electron reduction of molecular oxygen that produces superoxide as an initiating radical for generation of primary ROS followed by the mPTP induction. As we have already mentioned, the ROS burst accompanying ΔΨ collapse is against the existing “dogma” that ROS production is inversely related to the mitochondrial membrane potential magnitude (FIGURE 5). This dogma seems to be true when the classical uncoupling is considered, i.e., the drop of ΔΨ due to the enhanced proton conductance of the inner mitochondrial membrane. It seems to be irrelevant to the extreme dysequilibrium situation when a megachannel is opened in this membrane. The enhanced ROS production in mitochondria undergoing mPTP opening has been confirmed in vitro when isolated mitochondria were supplemented with NADH to compensate for its loss as a result of opening a megachannel (35).

Mitochondrial ROS rose simultaneously with the mPTP-induced drop of ΔΨ. However, quite often it has been possible to observe a very brief phase of mitochondrial hyperpolarization coinciding with the onset of the mPTP opening. The analysis proved that the hyperpolarization phase also coincided with the start of the excessive ROS generation (493) (FIGURE 9). Although a hyperpolarization spike has not been observed in all recorded mPTP opening events in the cardiac myocyte, the flicker could be sufficiently brief and be below the kinetic resolution of the fluorescent signal from TMRM belonging to the class of “slow-response” (distributed or accumulated) probes for the mitochondrial ΔΨ (458, 459). The flickering mitochondrial hyperpolarization mode was found to be a frequent phenomenon during photo-excitation of TMRM in loaded cardiac myocytes observed in the confocal microscope during line-scanning. Not every flicker could ignite the mPTP opening; nevertheless, each flicker initiated a small burst of ROS production that was insensitive to the mPTP inhibitor bongkrekic acid. Also these hyperpolarization flickers were not associated with the entry of the inert probe calcine from the cytosol into the mitochondria, suggesting these flickers do not involve a long opening of the mPTP. So, apparently there were at least two different modes of RIRR, one of which was accompanied by the large-conductor mPTP opening, while another was too brief to establish the conductance. It is noteworthy that the flicker-induced ROS production associated with mitochondrial hyperpolarization theoretically obeyed the conventionally established relations between ROS production and mitochondrial transmembrane potential (FIGURE 5) (240, 275), while the mPTP-associated ROS production did not. Both were caused by the triggering ROS formed during the photoexcitation process.
FIGURE 9. Transient Δψ hyperpolarization and depolarization (flickering) preceding MPT induction can be observed. A: overlay showing the 20-Hz linescan image in a cardiomyocyte loaded with TMRM (red) and DCF (green). B: the TMRM channel from A; arrows indicate examples of transient hyperpolarization immediately preceding Δψ loss (MPT induction). C: the directional derivative of the TMRM intensity with respect to time, to enhance the identification of intensity transients (transient hyperpolarization prior to MPT induction); arrows indicate examples of transient hyperpolarization (positive slope maxima) immediately preceding MPT. D: intensity plot of the TMRM responses of the mitochondrial pair indicated by the asterisk in B; inset shows an enlargement of the area inside the red box and shows transient (relative) hyperpolarization immediately preceding MPT. [From Zorov et al. (493), with permission from Elsevier.] E–G: ROS production (green) occurs during transient mitochondrial hyperpolarization flickering (red image, arrows in E). H–I: 2-Hz line scan image in a cardiomyocyte loaded with TMRM. Transient hyperpolarization and depolarization spikes are seen as white and black vertical lines (shown by the arrows from 1 to 7). TMRM fluorescence intensity (Δψ) in the region of the cardiomyocyte denoted by the arrow-bracket in H (~6 μm in width, consisting of 3 sarcomere-associated pairs of mitochondria) within which mitochondria display concerted flickerings of the membrane potential. Note that these transient depolarizations, during the time interval between arrows 1–7, are accompanied by progressive mitochondrial swelling (as seen by the lateral displacement of adjacent mitochondria) consistent with repeated transient MPT-induction episodes.
VII. RIRR AND Ca^{2+}

RIRR was named for the analogy with the process of Ca^{2+}-induced Ca^{2+} release (with acronym CICR) which has been known since the 1970s (125, 127, 128, 139) and which plays a critical role in excitation-contraction coupling. In this process, an action potential depolarizes the cell membrane providing a small influx of Ca^{2+} through the plasma membrane, which by itself is insufficient to provide contractile activation. Moreover, since the contractile apparatus is at a distance from the sites of inward Ca^{2+} transport, Ca^{2+}-induced muscle fibers contraction would be retarded if it depended only on Ca^{2+} diffusion within the muscle cell. CICR serves two goals. First, it significantly facilitates the propagation of the Ca^{2+} signal in the cell, and second, it amplifies Ca^{2+} concentration to reach Ca^{2+} levels in the vicinity of contractile apparatus essential to provide a contraction. Then, both RIRR and CICR contain signaling amplification loops to reach the threshold necessary for the transition. CICR and RIRR are also similar in spatial terms; CICR includes different intracellular compartments and covers the space between the plasma membrane Ca^{2+}-channels and ER/SR and fibers, and RIRR can span between mitochondria and thus spread across and between cells.

Although there have been some attempts in the past to ascribe mitochondrial mPTP to the existence of mitochondrial CICR (194) or even strontium-induced strontium release (190), eventually the essential role of ROS has been established in this process. In fact, mitochondria have quite a high Ca^{2+}-buffering capacity (maximal calcium uptake), explaining the fact that a number of consecutive Ca^{2+} pulses could be sequestered and tolerated by isolated mitochondria or permeabilized cells until mPTP opening occurs resulting in a robust release from mitochondria of both sequestered and intrinsic amounts of Ca^{2+} (or Sr^{2+}) (77, 166, 321, 331). Such mitochondrial ability to sequester Ca^{2+} could be modified by a number of factors (7, 30, 132, 306, 465).

Consequently, “X-induced X release” may be a general mechanism for any determined (such as RIRR and CICR) and still undetermined signaling amplification loops. Meanwhile, reciprocal amplification of ROS and Ca^{2+} signals or any other “X-induced Y” release processes representing solitary events or additional complex processes within a signaling cascade, probably also occur, e.g., in the case when ROS generated in mitochondria may be a source of Ca^{2+} unitary releases from ryanodine receptor of SR (Ca^{2+}-sparks) (491) (see FIGURE 10). The process of the spontaneous ROS-induced Ca^{2+} release by the SR ryanodine receptor described in Reference 491 has been recently analyzed in a model of sustained mitochondrial δΨ oscillations driven by the mitochondrially produced ROS. Dynamic changes in mitochondrial energy state resulted in altered frequency and properties of the Ca^{2+} sparks (485).

Isolated mitochondria possess an ability to sequester very large amounts of Ca^{2+} from the medium (85, 106, 259). Although proton motive force potentially drives cations to negatively charged mitochondrial matrix forcing cations to be accumulated inside of mitochondria, it has been found that at resting conditions in rat cardiac myocytes the intramitochondrial free Ca^{2+} level is <100 nM. Mitochondrial Ca^{2+} concentration is maintained by inward and outward Ca^{2+}-transporting systems in the inner mitochondrial membrane. One of these, the uniporter (231, 372, 450, 451), has low affinity but high capacity for transporting Ca^{2+}, whereas Ca^{2+}/2H^{+} and Ca^{2+}/2Na^{+} exchangers (60, 61, 75, 98, 136, 137) have much lower capacity. In stimulated cells at higher pacing rates, the increased cytosolic Ca^{2+} will steadily raise the [Ca^{2+}]_{im}. Over the course of many contractions, [Ca^{2+}]_{im} can rise up to 600 nM (300) and potentially results in activation of mitochondrial dehydrogenases. Pyruvate and α-ketoglutarate dehydrogenases show the greatest dependence on [Ca^{2+}]_{im} (107, 176, 278, 302). Such activation of the key mitochondrial dehydrogenases results in activation of respiration and ATP synthesis to meet rising energy demands under increased work load.

Interestingly, calcium ions target and activate those mitochondrial enzymes (pyruvate dehydrogenase and α-ketoglutarate dehydrogenase) that are considered to be a key source of ROS in mitochondria (see above). However, while the crucial role of Ca^{2+} in mitochondrial metabolism is established (reviewed in Refs. 166, 434) with recent molecular identification of the Ca^{2+}-uniporter (36, 105) and Ca^{2+}/2Na^{+} exchanger (332), the role of Ca^{2+} in ROS production in mitochondria remains controversial. In isolated mitochondria, the mPTP is opened by elevated Ca^{2+}. This led to the conventional assumption that mPTP induction by mitochondrial Ca^{2+} loading may be the cause of many

![FIGURE 10. Induction of Ca^{2+} sparks after the mPTP. Cell is dual-loaded with TMRM (ΔΨ) and fluo-3 (Ca^{2+}) and line-scan imaged at 230 Hz. Representative example showing the dissipation of TMRM fluorescence from a single mitochondrion and a cluster of Ca^{2+} sparks in the immediate vicinity, within seconds of MPT induction. Inset: comparison of Ca^{2+} spark rate in proximity of the mPTP occurrence, i.e., within the sarcomere containing the involved mitochondria and within 3 s after the mPTP occurrence. [From Zorov et al. (491).]]
types of cell death, such as those induced by ischemia-reperfusion injury and chemical toxins, in the heart and brain. In intact cardiomyocytes and neurons, however, normal excitability achieves Ca\(^{2+}\) elevations to levels comparable to those believed to induce the mPTP based on in vitro data. This suggests a serious paradox that will have to be reconciled regarding the role of Ca\(^{2+}\) in mPTP induction in vitro with the empirical evidence of health and longevity of these postmitotic cells in vivo (218, 494). Although Ca\(^{2+}\) is a typical tool for induction of permeability transition in isolated mitochondria (182, 195, 196; reviewed in Ref. 489), experiments with intact cardiac myocytes and neurons demonstrated that the mPTP is largely insensitive to increased cytosolic Ca\(^{2+}\) (see supplements to Ref. 218). In these experiments, however, after cell permeabilization, the Ca\(^{2+}\) sensitivity of the mPTP is unmasked and is similar to that observed in mitochondria isolated from these cells (FIGURE 11, A and B). Probably, some cytosolic factors that are lost after permeabilization and mitochondrial isolation are responsible for Ca\(^{2+}\) insensitivity of the mPTP in the intact excitable cell such as the cardiac myocyte or neuron. These facts themselves put into question the role of Ca\(^{2+}\) in cell death correlated with mPTP induction after, e.g., hypoxia/reoxygenation which may be overestimated due to limitation of the biological model (212) (see FIGURE 11C).

There is also a puzzling discrepancy between a very high apparent K\(_m\) (250–350 \(\mu\)M) for endogenous ADP in the control of mitochondrial respiration in permeabilized muscle cells and that in isolated mitochondria where the apparent K\(_m\) equals 15–20 \(\mu\)M (248). One of the possible explanations for this inconsistency is that isolated mitochondria are stripped from cytoskeletal proteins that maintain mitochondrial integrity within the intracellular energetic unit, which might be involved in fine regulation of enzymatic parameters in the live cell and be responsible for observed differences (22).

**VIII. MITOCHONDRIAL COMPARTMENTATION**

The reviewed data raise the question about the adequacy of isolated mitochondria or permeabilized cells to the actual processes taking place in an intact live cell, as all three objects have different levels of structural and organizational complexity. One of the structural parameters missing in isolated mitochondria is a close proximity of mitochondria to ER/SR found in the cell (119, 379, 384), thus making easier shuttling of small signaling elements (e.g., ROS, H\(^{+}\), or Ca\(^{2+}\)) and tightening the spatial and functional compartmentation of two organelles (357). The loss of spatial compartmentation due to the isolation procedure may dramatically change kinetics and, thus, enzymatic parameters of interactive systems. The compartmentalized system might greatly diminish the diffusion-controlled step(s) when the product of one enzymatic reaction almost immediately becomes a substrate of the second enzyme without the step of a product release in the bulk phase followed by extraction from this phase.

![FIGURE 11](https://example.com/figure11.png)

**FIGURE 11.** Ca\(^{2+}\) dependence of the mPTP in rat adult cardiac myocytes. A: mPTP ROS threshold during a “clamp” of intracellular Ca\(^{2+}\) >500nM for >20 min (using 10-Hz electrical tetanization in 5 mM bathing Ca\(^{2+}\) in the presence of thapsigargin or cyclopiazonic acid to disable the sarcoplasmic reticulum) is identical to that in unstimulated cells (with a cytoplasmic Ca\(^{2+}\) of ~100 nM). B: complete equimolar replacement of Ca\(^{2+}\) for Sr\(^{2+}\) (for 6 h) in intact cardiac myocytes resulted in the same mPTP ROS threshold as seen in cells with normal Ca\(^{2+}\). C: buffering intracellular Ca\(^{2+}\) (with BAPTA) or limiting Ca\(^{2+}\) influx (in nominally Ca\(^{2+}\)-free buffer) does not limit cardiac myocyte death after hypoxia/reoxygenation. [From Juhaszova et al. (218). Republished with permission from the American Society for Clinical Investigation; permission conveyed through Copyright Clearance Center, Inc.]
It is beyond the scope of this review, but the dramatic difference between two theories of oxidative phosphorylation, one proposed by Peter Mitchell and another by Robert Williams, deserves mentioning. Both theories were named chemiosmotic with one principal difference: while Mitchell’s theory considered the delocalized proton ejected from a proton pump into the bulk phase to be a substrate for ATP synthase complex (298, 299), Williams considered a localized proton to be consumed by ATP synthase without an intermediate step of going into the bulk phase (472, 473). Although there are some more or less sketchy observations supporting the second mechanism which apparently requires organization of supramolecular protein complexes (reviewed in Ref. 131), the first mechanism was generally accepted, and its founder was awarded the Nobel prize in 1978.

The problem of intramitochondrial compartmentation has many supporters (e.g., Refs. 412, 413; reviewed in Ref. 265). However, recent data has led to an interpretation highly critical of the organization of mitochondrial respiratory chain into supramolecular complexes (organized protein assemblies) (441), leaving the compartmentation issue still rather controversial.

Another important issue is the intermitochondrial compartmentation. The possibility that mitochondrial units may work in concert unifying energy transmission and signaling has been highly explored. Concerted work of individual mitochondria in striated muscles interconnected by electrically permeable junctions (28) was proposed (394) and later confirmed for cardiac myocytes (10). Such electrical unification of mitochondria was postulated to adequately allow fast transportation of energy along the mitochondrial reticulum to all cellular regions remote from the initial energy source. Later the mitochondrial matrix lumen continuity was confirmed by using photoactivated GFP (445), supporting the idea of mitochondrial organization in networks (115, 199, 395) which play an important role in intracellular signaling (46, 215, 395). Cooperation of mitochondria in terms of synchronous response to oxidative challenge as part of the RIRR process seems to open a new door for exploring alternative roles of mitochondria in the cell apart from their energetic function (491, 493).

**IX. MITOCHONDRIAL RIRR IN OSCILLATING MODE**

It is noteworthy that fluctuations of ROS within the cell in the vicinity of mitochondria can elicit instability of the intracellular redox state which, as we pointed out earlier, is greatly maintained by homeostatic mitochondrial functioning. **FIGURE 12** demonstrates ROS-induced fluctuations of the mitochondrial Δψ (one recorded in adult and another in neonatal cardiac myocyte) showing that mPTP in these cells can be reversed and the mitochondrial inner membrane be sealed and Δψ regained with many repetitive cycles of the mPTP induction and closure. The first demonstration in intact cells of such an oscillatory mode of the mPTP triggered by photodynamically induced ROS accompanied by the RIRR was given in 2000 (491) and further explored in 2006 (493).

The regulation of mitochondrial ROS production and their levels is exerted by a number of factors, such as the redox state of respiratory components, oxygen tension, ionic environment and the activity of redox buffers, etc. However, the mitochondrial environment may become unstable, which may cause the instability of mitochondrial functioning, and potential episodes of fluctuating mitochondrial ROS production are not an exception. Several researchers described synchronized mitochondrial oscillations including rhythmic changes of ion fluxes, respiration, and mitochondrial volume in vitro in suspensions of isolated mitochondria (particularly, under conditions of energized ion transport) (23, 83, 158, 168, 198, 312, 330). Typically, these oscillations had the form of damped sinusoidal rhythmic changes. Under these conditions, within each cycle, the mitochondrial shrinkage phase was associated with increased respiration, a more highly oxidized state of pyridine nucleotides, a stimulation of ATP hydrolysis, an inhibition of proton release, and stimulation of cation release. The shrinkage phase is followed by a swelling phase, and when
these changes were reversed, it also showed evidence of damping. It was concluded that the oscillatory states of electron and energy transfer pathways might be under the control of mitochondrial swelling and ion transport by a feedback mechanism.

Later, such oscillatory behavior of different mitochondrial parameters including those induced by ROS has been detected in intact cells and tissues (18, 92, 126, 281, 297, 360, 491, 493) showing that structural peculiarities in organization of mitochondria within the cell do not play the most important role in induction and propagation of oscillations.

X. IMAC-ASSOCIATED RIRR

Another model of RIRR was generated and explored in B. O’Rourke’s lab at Johns Hopkins University. The study was preceded by an investigation of oscillations in the sarcosomal current in cardiac myocytes caused by substrate deprivation resulting in spontaneous fluctuations of ATP-sensitive K+ current in parallel with changes of the redox state of NADH/NAD (324). This implies the critical role of energy in generating oscillations. Since metabolic oscillations were in concert with the shortening and suppression of the action potential, it has been concluded that these metabolic oscillations may be relevant to pathologies such as arrhythmias as a result of ischemia/reperfusion insult (reviewed in Refs. 20, 323). Later, it was found that a local two-photon excitation of cardiac myocyte covering a few mitochondria loaded with a mitochondrial membrane potential-sensitive fluorescent dye triggered oscillation of the membrane potential within these mitochondria. It also triggered the oscillations in intramitochondrial redox potential measured by a ratio of reduced-to-oxidized flavins. The initiating impulse was triggered by ROS generated by the local excitation resulting in production of primary ROS. The oscillations spread in three dimensions along all interconnected mitochondria, forming a lattice with the primary oscillators consisting of only a few mitochondria. These could later spread over the entire mitochondrial network and possibly extend beyond the boundaries of the single cell.

Mitochondrial inhibitors such as rotenone and bongkrekic acid suppressed mPTP-associated RIRR and ROS to the level below the threshold implicating the source of ROS as mitochondrial (491). However, these inhibitors as well as others including cyclosporine A, cyanide, myxothiazol, nigericin, and oligomycin did not block RIRR associated with transient mitochondrial hyperpolarizations (mitochondrial membrane potential flickers described above) (FIGURE 9) (493). In contrast to mPTP-associated RIRR, in the RIRR mode associated with hyperpolarization flickers, the brief changes of the membrane potential were not accompanied by significant redistribution of an inert fluorescent agent of 620 Da (calcein) between mitochondria and cytosol. Therefore, the mPTP is either too transient for sufficient movement of calcein to be measured, or of low conductance, or possibly even not involved in this type of oscillatory mode. In a comprehensive search for ion channels responsible for the various types of non-mPTP-related mitochondrial oscillations, the focus moved to the so-called IMAC because the oscillations were blocked by the IMAC inhibitor DIDS (38–40). In addition, these oscillations were significantly attenuated by ligands of the mitochondrial (peripheral) benzodiazepine receptor (PBR) such as Ro5–4864 and PK11195 (18, 323). It has been speculated that PBR (which presumably consists of ANT, VDAC, and 18-kDa protein of the outer mitochondrial membrane, TSPO) could be responsible for IMAC (20) since some ligands of PBR can block the mitochondrial inner membrane 107-pS channel (230) which has moderate anion selectivity (410). However, PBR ligands (230) as well as the mPTP inhibitor cyclosporine A (430) both block mitochondrial giant MCC (multiple conductance channel), implying that mPTP might be related to the functional state of PBR. However, this speculation needs additional experimental support with elucidation of the molecular identity of IMAC.

Thus, in addition to mPTP-associated RIRR, another independent mode was proposed for RIRR which involves IMAC. According to this model, mitochondrial respiratory chain is the main oscillatory source of ROS which can be released to the cytosol through IMAC due to its permeability for superoxide (449) produced in complex III (FIGURE 13).

Recently, the Δψ oscillations and RIRR induced by local oxidative stress (401) and by perfusion with hydrogen peroxide (53) have been detected in live, intact myocardium. Perfusion of rat hearts with hydrogen peroxide, depending on the concentration of the oxidant, elicited two peaks of superoxide in live myocardium measured by optical mapping of the fluorescent ROS probe, with the second peak being much more intense than the first one (53). Spatiotemporal ROS mapping during the second ROS peak revealed a propagation of superoxide signal within the cardiac tissue with a velocity ~20 μm/s. The hearts with the second peak displayed a much greater arrhythmia compared with those where the second peak was absent. PBR ligand, Ro5–4864, and superoxide dismutase mimetic but not cyclosporine A abolished RIRR and ventricular tachycardia and ventricular fibrillation, suggesting IMAC involvement. These findings further extended the concept of RIRR as a key factor in the incidence of postischemic arrhythmias.

A computational model with superoxide as a trigger of mitochondrial Δψ depolarization has been developed (484, 486). The model is based on the percolation theory to explain how neighbor-to-neighbor interaction defines propagation of the signals along the spatially organized excitable matrix, arising from a spanning cluster of oxidatively
stressed mitochondria united in the network (19, 20). In addition, the dynamic spatiotemporal properties of individual mitochondrial oscillators within stress-induced synchronized oscillatory clusters of cardiac mitochondrial network have been characterized by applying wavelet-based analysis (252, 253). Individual oscillating mitochondria within clusters have been identified and their frequency correlated with the size of the cluster. As the cluster grew larger, they required more time to achieve full synchronization of all mitochondria within the cluster. Additionally, the percentage of cluster size has been inversely correlated with the percentage of amplitude. Nonlinear characteristics of the mitochondrial network, particularly in the heart, make mitochondria prone to the local disturbances including those involving oxidative stress. Once these local disturbances are formed, they spread over the network causing loss of its normal functioning. They cause a mismatch of the finely tuned balance of available energy production to energy expenditures (93, 365, 435, 479), resulting in an inability of mitochondrial network to completely repolarize between oscillations. Finally, it reaches a point of no return that results in pathologies and ultimately cell death.

XI. RIRR-RELATED PATHOLOGIES: THE IMPORTANCE OF Δψ HOMEOSTASIS

Mitochondria are not only the energy source but also one of the primary sources of a vast number of deadly pathologies. As to cardiovascular problems, a striking example of “misbehavior” of a mitochondrial network extending over the whole heart and affecting the whole organism is ischemia/reperfusion-induced oxidative injury which in extreme cases results not only in cell death, but also organ failure, and eventually organismal death. Experiments with single cardiac myocytes exposed to transient hypoxia, followed by a reoxygenation phase, demonstrated substantial mitochondrial instability caused by the introduction of oxygen into the system. In these cells, transient mitochondrial hyperpolarization was followed by a progressive rise of the ROS level in deteriorating mitochondria to the point of mPTP introduction and accompanying large ROS burst (FIGURE 14A), i.e., typical of RIRR. The incidence of cardiac cell death depended on the proportion of damaged mitochondria having developed mPTP to their total number in the cell and on the physiological status of the cell (218) (FIGURE 14B).

To assess the degree of system readiness to resist an oxidative challenge, a simple approach was developed using time as a factor. Time quantifies the titrated amount of ROS (delivered incrementally by successive photoactivated exposures during linescan imaging) required to achieve the threshold for mPTP induction in a particular mitochondrion (218, 491). Apparently, mitochondria with higher resistance to oxidative stress require a longer time (i.e., higher total oxidant exposure level) to open the pore while those with a low tolerance require shorter time (i.e., lower total oxidant exposure level) for the mPTP induction. The timing of mPTP opening (t_mPTP) is an integral factor, depending on the rate of oxidant quenching (the level of intramitochondrial and extramitochondrial antioxidants, e.g., glutathione) and the physicochemical state of the system responsible for the generation of the mPTP as an event. While the molecular identity of the mPTP needs to be validated in detail (157, 217, 494), the presence of bcl-2/bcl-xL or phosphorylated glycogen synthase kinase 3 (P-GSK-3) in the vicinity of the pore or attachment of hexokinase II desensitize the pore to oxidants (86, 217, 218) and extend the time necessary to overcome the desensitization and open the pore. The conventional line-scan confocal microscopy has been employed as a model to determine the ROS threshold for inducing the mPTP. For this purpose, an ideal experimental object is a
cardiac myocyte with mitochondria arranged in regular lattice-like arrays rendering the line-scan instrumentation straightforward (491, 493, 496) (FIGURE 15A). Furthermore, the same approach has been successfully applied to neuronal mitochondria in situ and cultured neonatal cardiac myocytes (217) and potentially could be applied to mitochondria in other cell types with cell motility or intracellular mitochondrial motion during the line-can procedure being carefully considered.

It is not surprising that the mPTP threshold is highly dependent on ambient molecular oxygen, since ROS production in the system is a reaction of the first order with respect to molecular oxygen. In cardiac myocytes incubated in media with low O2, \( t_{mPTP} \) was much greater than in mitochondria incubated in normoxic or hyperoxic media (FIGURE 15B). Consequently, adequate oxygenation of cellular mitochondria is important for unambiguous interpretation of \( t_{mPTP} \).

The value of \( t_{mPTP} \) determined by the above-mentioned approach, the inherent resistance of mitochondria to mPTP induction by ROS, and can be significantly prolonged by ischemic or pharmacological preconditioning. This approach has been applied for the detailed elucidation of the architecture of the signaling pathways engaged by ischemic/pharmacological preconditioning (218, 385).

**FIGURE 14.** ROS are involved in mitochondria and cell deterioration. A: hypoxia/reoxygenation-induced mitochondrial hyperpolarization leads to increased ROS. Mitochondria stained with TMRM (Δψ, red) and DCF (ROS, green) were laser line-scanned (2 Hz) during hypoxia and the reoxygenation phase. The ROS burst is delayed after reoxygenation and starts at the maximum Δψ. Mitochondrial hyperpolarization lasts for ~2 min, followed by loss of Δψ. B: cell survival after constant-energy photoexcitation of a 25 x 25 μm² region. Right panels show TMRM-stained cardiomyocyte (red) immediately after, and 1 h after, irradiation. Survival is inversely related to the fraction of mitochondria (mito) undergoing mPTP induction and is improved by ROS scavenger (Trolox), NO donor (SNAP), and the K<sub>ATP</sub> channel opener diazoxide (Dz) and is impaired by 5-hydroxydecanoate (5HD), the blocker of K<sub>ATP</sub> channel. [From Juhaszova et al. (218). Republished with permission from the American Society for Clinical Investigation; permission conveyed through Copyright Clearance Center, Inc.]

**FIGURE 15.** Linescan technique applied to mitochondria in rat cardiac myocytes. A: definition of the mPTP ROS threshold as an average time over population of mitochondria (shown by a dashed red line) in myocyte stained with fluorescent mitochondrial probe for the membrane potential to induce the mPTP (shown by arrow) (491, 493). B: dependence of the mPTP induction in the cell on ambient molecular oxygen.
The term *mitochondrial criticality* was coined (16, 17) for the situation when the mitochondrial network in the cardiac myocyte becomes highly sensitive to the changes of ambient/intramitochondrial ROS, for example, by responding as synchronized oscillatory depolarizations of the mitochondrial Δψ which could be scaled to the whole cell or cluster of myocytes (401). When the mitochondrial system reaches the point of criticality, oscillations of Δψ are ignited, eventually creating spatial and temporal heterogeneity of excitability in a heart exposed to oxidative challenge, e.g., under hypoxia/reoxygenation. This inflicts dramatic pathological changes to the heart involving lethal cardiac arrhythmias possibly followed by cardiac arrest and an individual’s death.

Based on the discussion above, we would tentatively suggest the existence of specific in vivo mitochondrial states that may contribute to the fate of the cell (see FIGURE 16). The relatively stable state (state A) has a comparatively high tolerance to oxidative stress and is able to sustain the Δψ for a longer time (with longer $t_{\text{MPT}}$). This is characteristic of the healthy cell where the MPT formation is delayed. States B and C characterize mitochondria which have been stressed (prior to experimental procedure of the MPT-induction protocol has been employed) and thus are unable to sustain the Δψ for an extended period. In both cases (B and C), the first mean $t_{\text{MPT}}$ value is similar, characterized by a shorter $t_{\text{MPT}}$ compared with mitochondria in state A, but there are some obvious and significant differences between the states B and C. While in state B mitochondria remain depolarized after MPT induction, mitochondria in state C are characterized by dynamic instability revealed by Δψ oscillations which progressively deteriorate and ultimately cease. These differences probably reflect not only differences in the state of the antioxidant defense system in B versus C but also intrinsic differences in the function/activities of the pore complex subunits itself triggered in response to posttranslational modifications, the signaling status of the immediate pore modulators, and/or changes in the pore complex arrangement at the protein level (FIGURE 16).

A preponderance of data point to the fact that mitochondrial membrane potential is an essential attribute of mitochondria, and its homeostasis is a prerequisite for the health of mitochondria and the preservation of normal cell and tissue function. Under hypoxic conditions, when mitochondria are incapable of sustaining ΔΨ driven by respiration, the mitochondrial membrane potential is maintained at the expense of cytosolic ATP hydrolysis by mitochondrial ATPase (110). Sufficient buffering of the mitochondrial Δψ by cytosolic ATP may explain why the intracellular concentration of ATP (a few mM) exceeds the $K_m$ for intracellular ATP-utilizing enzymes by orders of magnitude. Thus, under physiological conditions, these enzymes are fully saturated and do not depend on ATP concentration for their function.

The homeostasis of mitochondrial Δψ is critical for the mitochondrial function. Only small vital variations in a range (approximately between 160 and 220 mV) were tolerated in isolated mitochondria at states 3 and 4 according to the terminology of B. Chance (82). These small variations pre-

![FIGURE 16. Basic mitochondrial redox states (see explanation in the text). A: unstressed mitochondria with high tolerance to ROS (high survival). B: stressed mitochondria with low tolerance to ROS (low survival). C: stressed mitochondria with high tolerance to ROS (unknown survival).](image-url)
serve the minimal energy that may be sufficient for phosphorylation of ADP by mitochondrial ATP synthase but may dramatically influence ROS production within mitochondria (see FIGURE 5). In case of a drop of the membrane potential below the phosphorylating membrane potential, especially when it is nulled after induction of mPTP, mitochondria could initiate a process of self-destruction involving Ca\textsuperscript{2+}-independent degrading hydrolases and phospholipases (66, 261, 495, 496). This process is called mitophagy (reviewed in Refs. 88, 234, 258) and further substantiates the vital importance of maintaining Δψ homeostasis to keep mitochondria structurally and functionally intact. The questions regarding a mitochondrial Δψ sensor, or which processes in mitochondria could be considered as vital, the cessation of which will result in mitochondrial degradation, remain unknown. One possible candidate for such a process is the energy (Δψ)-dependent transport of peptides into mitochondria (reviewed in Ref. 338). This machinery is highly important for the quality control of mitochondria (362) through the Δψ-dependent ubiquitin labeling of the impaired mitochondrion resulting in the initiation of mitophagy (see explanations in the FIGURE 17; Ref. 210).

**XII. PRACTICAL ASPECTS OF USING MITOCHONDRIAL Δψ AS A DRIVER AND REPORTER**

The mitochondrial membrane potential drives natural cationic substances into mitochondria, and this property could
be utilized for intramitochondrial transport of man-made drugs.

In principle, the delivery of drugs to the mitochondrial interior can be executed by using two targeting vehicles: 1) mitochondrial targeting signal sequences and 2) mitochondrial protein import systems. While the first approach implies specific transport of the vehicle through the TIM/TOM mitochondrial protein transport system (318), the second uses positively charged lipophilic compounds, for example, constructed in a way to delocalize the charge over the set of coupled double bonds (268). Due to negative charge of the mitochondrial interior, the latter nonspecifically accumulates these cations to the thermodynamically permissive level which theoretically exceeds extramitochondrial levels by about three orders of magnitude. The idea of driving such charged compounds by virtue of the electrogenic nature of mitochondrial energetics is fully consistent with Mitchell’s chemiosmotic theory (268, 298, 299). Nowadays, after establishment of this mechanism (1), the approach started to serve two experimental missions: to deliver to the mitochondrial interior a desired compound which is bound to a mitochondrialotropic vehicle (225, 393, 407) and, on the other hand, to discriminate between the mitochondrial and nonmitochondrial nature of some process under study. In this context, mitochondrialropic antioxidants are not only potentially beneficially targeted drugs to quench unwanted excessive ROS and other reactive species in mitochondria, but they may also serve as excellent reporters providing an answer as to whether a given mechanism involves mitochondrial ROS. In the first case, in vitro data and animal experiments have suggested the possible beneficial effect of mitochondria-targeted antioxidants (such as MitoQ, SkQ1, SkQR1, and others) in treatment of a great number of model systems associated with oxidative stress. These include stroke, arrhythmias, ischemia/ reoxygenation, chemical toxicity, infection, inflammation, and some inherited and acquired age-related and unrelated diseases (such as Alzheimer’s, diabetes, hepatitis, metabolic syndrome, hearing loss, etc.) (14, 27, 146, 206, 223, 236, 269, 292, 307, 343, 388, 391, 399, 404–406, 408).

Apart from the practical issue of using mitochondrion-targeted chimeric compounds as potential therapeutic agents, their specific effects uncovered the fundamental role of mitochondria and mitochondrial ROS in the onset and propagation of different pathologies. What we have learned is that ROS originating from mitochondrial rather than from other intracellular sources, when they exceed the homeostatic level, are among the most pathogenic factors (222, 382, 383, 437, 467). In this context, targeted normalization of ROS levels (specifically in mitochondria) may be beneficial in preventing a number of ROS-related pathologies including that involving RIRR (310, 392). The requirement of mitochondria-derived ROS for the propagation of RIRR together with its concomitant pathological cell death has received support from experiments wherein 1) the primary extramitochondrial ROS source induced a secondary intramitochondrial ROS release, and 2) both of these processes were prevented by specific mitochondria-targeted antioxidants (344).

**XIII. CONCLUDING COMMENTS**

ROS-induced ROS release is a fundamental process originating in mitochondria (18, 491, 493) responding to an increased oxidative stress by a positive feedback loop resulting in a regenerative, autocatalytic cascade which in a vast majority of cases is terminated, presumably by the local redox environment together with a change of functional properties of the “release valve.” Indeed, for this mechanism to serve a physiological role, its underlying properties of chain-reaction-like propagation must be efficiently terminated to prevent the unwanted spread and widespread destruction of mitochondria risking unintended and unwanted loss of the cell. These considerations are entirely analogous to the control of a nuclear chain reaction in power plants (e.g., by control rods) or that of CICR in excitable cells by intrinsic ryanodine receptor- and SR-related mechanisms (423). When RIRR is appropriately not terminated, it may lead to the wanted removal of a mitochondrial or cell, e.g., damaged beyond repair. When RIRR is inappropriately not terminated, it may lead to unwanted cell loss such as after stroke or myocardial infarction.

We propose that RIRR may result in a cellular response that can be considered either adaptive (e.g., promoting signaling resulting in the elevation of the tolerance to oxidative stress or the removal of unwanted organelles and cells) or maladaptive (e.g., the pathological destruction and death of functional mitochondria and vital, essential cells). Adaptive cell elimination may involve cell death executed by necrotic or apoptotic mechanisms (for example, elimination of non-functional or damaged cells). It remains an open question whether RIRR mechanisms would be recruited to serve the adaptive elimination of cells necessary to achieve proper organ size, shape and functions related to embryogenesis, self-antigen-recognizing immune cells that would be dangerous, cells no longer needed (e.g., nonlactating breast in- volution after pregnancy), activated lymphocytes after infection is resolved, or pre-cancer and cancer cells.

The RIRR phenomenon provides an opportunity to see the mitochondrion as machinery designed not only to trigger but also to facilitate important signaling mechanisms, further extending the concept of the mitochondrion as an excitable organelle capable of generating and conveying redox, electrical (10), and Ca2+ (199) signals. Therefore, mitochondria are not only efficient cellular energy powerhouses, they are significant signal transmitters and signal resonators, by which we mean the mitochondrial ability to...
respond to and to enhance, internal and external signals, in a manner analogous to the propagation, and \( \text{Ca}^{2+} \)-activated reaction triggering of CICR (125, 127, 128, 139).

We propose that the mPTP may be involved in at least four conceptual modes of pathophysiological function. The first role is to serve as a potential release valve which transiently opens a gate for controlled release of accumulated ions or newly formed low-molecular-weight toxic substances including elevated ROS and \( \text{Ca}^{2+} \). This likely maintains mitochondrial (and cellular) redox homeostasis, which is a critical point in normal cell functioning. The characteristics of this mode include brief and reversible episodes of the mPTP pore opening or activation of some other mitochondrial pores/channels/leaks constituting a low-conductance state of the mPTP which have been demonstrated in in vitro systems (5, 29, 244, 255). We conceive that this serves a housekeeping function, and at low energy demand (rest), it is likely relatively inactive. At high stress/energy demand conditions, this function plays a significant role (123). This is borne out by the study (124) whereby the genetic loss of CyPD (Ppif

result in elevated resting levels of \( \text{Ca}^{2+} \) presumably due to an inappropriate high “set-point” (FIGURE 17), which may reinforce the transition to irreversible mPTP opening (i.e., the point of no return) (88, 188). The timing to reach this point is controlled in part by the depletion of the intramitochondrial redox buffer caused by the flux of external (or internally produced) oxidants (491). This point may correspond to the term mitochondrial criticality (17) by reaching a redox crisis in an oxidatively stressed cell after which the behavior of mitochondrial energetics becomes unstable. By this principle, criticality determines ROS-induced organization of an excitatory mitochondrial cluster spreading oscillations of \( \Delta \psi \) over the cell.

The fourth mode of the mPTP is the terminal destructive step resulting in the elimination of the cell (which could be wanted or unwanted). As for mitochondria, impaired cells can be programmed for the destruction with the mPTP playing a critical role in that process. In general, this mechanism of cell elimination would encompass a large fraction of mitochondrial, undergoing the process described above, involving significant releases of ROS from mitochondria undergoing mPTP (218).

Schematically, FIGURE 18 represents a hierarchical model of the proposed pathophysiological roles and functions of the mPTP. According to this model, the stable state with the closed mPTP can undergo a transition to a metastable (transient) state of the mPTP. By analogy to an energy barrier (threshold) necessary to overcome in order to undergo a physico-chemical transition, the reaction probability and rate is determined by a redox threshold (process I) that normally is lower than the threshold of process II determining the transition to the fully open, stable mPTP. Such a metastable state of the pore (preceding its potential transition to a long open state) occurs as a result of intra- and extramitochondrial changes of the redox buffer still compromising the dynamic balance between the influx/production of oxidants and antioxidants, and the stability of the “gating architecture” of the mPTP complex. As discussed above, the RIRR-mPTP process may serve to provide an adaptive release of excess \( \text{Ca}^{2+} \) and ROS from mitochondria. The latter may be responsible for redox-activated pro-

The third mode constitutes a persistent, generally irreversible mPTP-associated large conductance of the inner mitochondrial membrane sufficient to activate the mechanisms of destruction of mitochondria destined for elimination (for example, by mitophagy, because they do not meet the necessary mitochondrial “quality control” standards). Apparently, the existence of impaired mitochondria could be a threat for the proper functioning of the whole mitochondrial population (because they are connected in a functional network), and opening of the mPTP may be interpreted as the switch leading to programmed mitochondrial elimination (mitophagy) (66, 76, 88, 210, 234, 258, 261, 446, 495, 496). The signal initiating the process of mitochondrial excitation may arise from the consistent and long-lasting drop of the mitochondrial membrane potential (210) (FIGURE 17), which may reinforce the transition to irreversible mPTP opening (i.e., the point of no return) (88, 188). The timing to reach this point is controlled in part by the depletion of the intramitochondrial redox buffer caused by the flux of external (or internally produced) oxidants (491). This point may correspond to the term mitochondrial criticality (17) by reaching a redox crisis in an oxidatively stressed cell after which the behavior of mitochondrial energetics becomes unstable. By this principle, criticality determines ROS-induced organization of an excitatory mitochondrial cluster spreading oscillations of \( \Delta \psi \) over the cell.
Protective signaling. The stable-open mPTP state is a result of the redox transition to the point of no return (long-lasting mPTP-opening) leading to the destruction of mitochondria, and when involving a sufficient fraction of the entire mitochondrial population, it results in the death of the cell. The height of both ROS thresholds (FIGURE 18) is under redox control as well as the sensitivity of the mPTP-gating mechanism (which is regulated by certain enzymatic posttranslational modifications), both of which may be targets for pharmacological intervention. It has been shown that the height of the second threshold is age dependent, being higher in mitochondria of young and lower in mitochondria of old animals. Various mPTP-related cytoprotective signaling cascades (e.g., through preconditioning, activation of PKC, receptor triggers including adenosine, opioid and β-receptors, mitochondrial ATP-dependent potassium channels, inhibition of glycogen synthase kinase-3β, and other effectors; described in Ref. 218) as well as more enhanced redox buffering (491), high deacetylase activity, specifically resulting in deacetylation of CyPD (387), attachment of hexokinase II to the mPTP complex (86, 333) restore or even drive this threshold up, but this protective signaling also fails with aging (reviewed in Ref. 216). Therefore, it is possible that the beneficial “anti-aging” effect of increased deacetylation (191) (particularly by mitochondrial SIRT3–5; Refs. 43, 160, 429) of critical mPTP-regulatory elements such as CyPD (172) may partially be explained by a relative desensitization of the mPTP complex to ROS induction which could restore the age-dependent decline of mPTP ROS threshold back towards to the basal level (“young” state).
There is new information concerning the identification of ATP synthase complex as the core structure necessary to form the mPTP that is certain to be relevant to the RIRR mechanism. The important role of mitochondrial ATP synthase complex (complex V) in the mPTP functioning has been proposed in a number of studies over more than two decades (56, 87, 197, 322), in part based on the fact that oligomycin prevents mPTP in isolated mitochondria (321). However, this fact was explained by a probable indirect effect of changed levels of adenine nucleotides rather than by a direct effect on the pore structure itself. Recent evidence indicates that a dimer of complex V is necessary and sufficient to form the mitochondrial pore with the apparent modulating role of CyPD (157). It is tempting to speculate how this could potentially confer the gating mechanism of the mPTP and explain bimodal behavior of ATP synthase in terms of induction of the mPTP. Such bimodality (opened/closed pore) may correlate with a bimodality of ATP synthase (dimer/monomer and higher order oligomers). Quick and sometimes reversible transitions from closed to open state of the pore (mPTP oscillations or flickers) may be speculated to stem from a bimodal output of CyPD enzymatic activity which is a rotamase (peptidyl prolyl cis-trans isomerase; Refs. 135, 431) determining two modes of conformation of proline residues in the targeted protein(s) (134) (e.g., such as ATP synthase complex dimer), forming the mitochondrial pore. Additionally, this may also explain why CyPD acetylation critically appears to be affecting this process, for example, during aging (387).

In addition to the mPTP-associated RIRR, the important contribution of the IMAC-associated mode of RIRR functioning (20, 323), especially in the mitochondrial network propagation properties, and its possible role in arrhythmogenesis, needs to be established. Resolving the molecular identity of the IMAC will enable us to better appreciate its role in physiology and pathology. It seems that IMAC-associated redox instability during pathological stress is quite different from a safe, physiological flickering mode of the mPTP, and it is possible that IMAC-related mechanisms may contribute to a transitional state between mPTP-associated signaling and pathological modes of RIRR.

In conclusion, mPTP and IMAC are the main known players in the mechanism of RIRR which is a multifaceted process important in the physiology and pathology of the cell. The mitochondrial role in this mechanism is above and beyond its well-recognized energy-producing activity, but includes signaling potentially regulating both positive and negative destructive roles. Mitochondrially produced ROS are elements that may activate/deactivate a diverse set of signals igniting as well as terminating signaling cascades. Apparently, the “fine-tuning” regulation of ROS levels in mitochondria is an essential function of RIRR, and mitochondria are important players in the propagation of ROS signals within a cell. Hypoxia/ischemia raises basal production of ROS which may activate the mPTP and/or IMAC and, if not appropriately terminated, is involved in the conversion of signaling to pathological ROS. The primary ROS signal to ignite RIRR may originate in as well as outside of mitochondria and may in turn be amplified by the RIRR mechanism. The ROS amplification site may coincide with one or more already known mitochondrial sites of ROS production. The full understanding of the RIRR mechanism will require a more comprehensive and detailed mechanistic knowledge of the ROS-producing sites operating in situ and in vivo. It is certain that at least some of the mechanisms of mitochondrial ROS production presented in this review are relevant to both physiological and pathological ROS production in the cell.

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MITOCHONDRIAL ROS AND ROS-INDUCED ROS RELEASE


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