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# The Role of Mitochondria in Reactive Oxygen Species Metabolism and Signaling

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## Abstract

Oxidative stress is considered a major contributor to the etiology of both "normal" senescence and severe pathologies with serious public health implications. Several cellular sources, including mitochondria, are known to produce significant amounts of reactive oxygen species (ROS) that may contribute to intracellular oxidative stress. Mitochondria possess at least 10 known sites that are capable of generating ROS, but they also feature a sophisticated multilayered ROS defense system that is much less studied. This review summarizes the current knowledge about major components involved in mitochondrial ROS metabolism and factors that regulate ROS generation and removal at the level of mitochondria. An integrative systemic approach is applied to analysis of mitochondrial ROS metabolism, which is "dissected" into ROS generation, ROS emission, and ROS scavenging. The *in vitro* ROS-producing capacity of several mitochondrial sites is compared in the metabolic context and the role of mitochondria in ROS-dependent intracellular signaling is discussed.

### Keywords

ROS sink; ROS targets; ROS sources; nutraceutics; succinate; malate; hypoxia-induced ROS increase

## Introduction

Ever-accumulating evidence supports multiple important roles of reactive oxygen species (ROS) in cell metabolism and signaling. The significance of ROS as aggravating or primary factors in numerous pathologies and senescence is firmly established, widely recognized, and extensively reviewed elsewhere (e.g., see Refs. 1–13). More recent data strongly suggest that ROS are involved in physiological signaling cascades regulating various cellular and organ functions,[9,14–16] with $H_2O_2$ being a chief messenger molecule. This is a relatively new and rapidly expanding research field so it is not surprising that many aspects of cellular ROS metabolism are not yet well understood. Mitochondrial ROS metabolism represents one of such difficult issues. This minireview summarizes the current knowledge about major components involved in mitochondrial ROS metabolism and factors that regulate ROS generation and removal at the level of mitochondria. We also present some of the most recently published and unpublished experimental data obtained in our and other laboratories. Some of this material has been earlier presented by the author at The New York Academy of Science



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## Are Mitochondria a Source, a Sink, or a Target of ROS?

For the purpose of this review, mitochondria in all their complexity can be viewed as being constructed of two functionally distinct "blocks," the "Catabolic engine" (CE) and the "ROS defense system" (RDS) (Fig. 1).[17] The former includes all enzymatic systems catalyzing the oxidation of various substrates generated inside and outside mitochondria, such as the tricarboxylic acid cycle, β-oxidation of fatty acids, and other enzymes. All the reducing equivalents generated in these reactions reduce pyridine and flavin nucleotides NAD and FAD, which in turn are oxidized by coenzyme Q (CoQ) in reactions catalyzed by several enzyme complexes located in the inner membrane of mitochondria. The flux of electrons from substrates through various redox carriers and centers in these enzymes is ultimately terminated in a four-electron reduction of molecular oxygen to water, catalyzed by the cytochrome *c* oxidase (Fig. 1). The current paradigm holds that *en route* to water some of the substrate-derived electrons are diverted from the flow and participate in a single-electron reduction of oxygen, thereby converting it into superoxide, a progenitor ROS. *This renders mitochondria a source of ROS*.

The RDS comprises several enzymes specialized in removal of superoxide, $H_2O_2$, and organic hydroperoxides (Fig. 1). Most of these enzymes are ubiquitously present in all mammalian mitochondria; the expression level of these enzymes exhibits tissue and species specificity (see Ref. [18] for details about individual enzymes pictured in Fig. 1). A unique feature of RDS is that almost all its enzymes rely on NADPH as a source of reducing equivalents needed for their activity. The NADPH reduction is carried on by three intramitochondrial enzymes: isocitrate dehydrogenase (NADPH linked), malic enzyme, and transhydrogenase.[19] These enzymes utilize substrates that are either shared, or generated, or both in the reactions catalyzed by CE. To note, the intramitochondrial pools of NADPH and GSH are rather large (ca. 3–5 mM NADPH20،21 and 2–14 mM GSH22¯25). Due to this, transient changes in the activity of CE would not immediately affect the amounts of NADPH and GSH available to RDS and its ability to extinguish short bursts in ROS concentration, its "shock-absorbing capacity." However, a prolonged activity of RDS, its "endurance," ultimately depends on the supply of NADPH and GSH, thus depending on the ability of CE to regenerate these compounds. Another unique feature of RDS is that it is not specific toward dealing with intramitochondrial ROS; exogenous ROS species capable of crossing the inner membrane of mitochondria are also detoxified by RDS. Moreover, it is done at the expense of some reducing equivalents generated in CE. *This renders mitochondria a sink of ROS*.

A feedback relation between the two systems is further amplified by the fact that some crucial components of CE, such as isocitrate-generating aconitase26 and a key trichloroacetic acid (TCA) enzyme ketoglutarate dehydrogenase complex,27 are very sensitive to the damage caused by ROS. Their damage could result in the overall impairment in the catabolic flux paralleled by a decrease in the efficiency of RDS because of the lack of NADPH and an increase in the intramitochondrial ROS. Recalling that mtDNA is particularly vulnerable to ROS-induced damage (e.g., Refs. 28¯30) and that it encodes all mitochondrial tRNAs and ribosomal RNAs and 13 of the mitochondrial proteins crucial to the proper functioning of the mitochondrial CE, which in turn supplies ATP to the cell and performs numerous other tasks crucial for sustaining cell viability, one might also think about *mitochondria as primary targets of ROS-induced cellular damage.*

There of course is a fourth possibility; pertinent to circumstances, mitochondria might behave as a source, sink, and target of ROS simultaneously or switch between these three modes

depending on the spatial and temporal metabolic conditions in their surroundings. Further insight on the role of mitochondria in ROS metabolism may be gained by comparing the capacities of CE and RDS toward ROS production and removal and the regulation of these systems by metabolic signals.

## Characteristics of the Mitochondrial ROS-producing System

Recalling that superoxide/$O_2$ couple has a moderate redox potential ($E_{1/2}$ = −0.16 V31), the reaction of one-electron reduction of oxygen is thermodynamically favorable for numerous mitochondrial oxidoreductases.[32] Therefore, determining a "main" source of ROS in CE appears to be difficult, if possible at all. Nevertheless, an evaluation of ROS-producing capacity of individual parts of CE is possible, to a degree, in experiments with isolated intact mitochondria and their subfractionated components. By using this approach, 10 potential sources of ROS have so far been identified in mammalian mitochondria (Fig. 2 and see Refs. 18, [33] for details). Among these sources, the highest ROS-producing capacity has been demonstrated for Complex I and Complex III (Table 1) of the mitochondrial respiratory chain and the enzyme dihydrolipoamide dehydrogenase. The latter enzyme is the common component of pyruvate, α-ketoglutarate, and branched-chain ketoacid dehydrogenase complexes and also participates in the glycine-cleavage system.[34] In intact mitochondria, their activities are linked to each other and to the rest of CE through the common pools of intermediates, such as NADH and CoQ. A change in electron flux through one of them changes the flux through the other electron carriers in CE. In other words, the degree of reduction of electron carriers in these enzymes in intact mitochondria is determined by the overall flux of reducing equivalents through the CE. An important ramification of this is that ROS production capacity of mitochondria should be controlled by the factors affecting and reflecting the metabolic state of intact mitochondria. It has been found that the most important factors controlling the ROS production in mitochondria are the chemical nature of the substrates fuelling CE, the amplitude of the membrane potential in mitochondria, the pH of the matrix of mitochondria, and the oxygen tension in their surroundings.

### Metabolic Factors Affecting Mitochondrial ROS Production

Although many substrate dehydrogenases comprising CE use a common pool of NAD as their electron acceptor, they exhibit quite different structural, physical, and chemical properties, such as the midpoint redox potentials and the rates and equilibrium constants of the catalyzed reactions. Because of this, a degree of reduction of NAD pool ($NADH/NAD^+$ ratio) under a steady-state condition depends on the chemical nature of the oxidized substrates. In turn, it affects the steady-state reduction level of other electron carriers linked to this pool in CE and the rate of ROS emission. It has been demonstrated that the rate of ROS emission by isolated mitochondria oxidizing NAD-linked substrates obligatory depends on the choice of substrate and almost linearly correlates with the characteristic NADH reduction level set by the oxidation of a selected substrate.[35,36] The rate of ROS production increases at high $NADH/NAD^+$ ratios.[18,33]

Succinate represents a special case of all the metabolites oxidized in CE. It has long been known that succinate oxidation can support the highest rate of ROS production in non-phosphorylating mitochondria (Fig. 2; Ref. [18] and references therein). However, many researchers dismissed the physiological significance of this fact on the grounds of both the perceived molecular mechanism of the effect and low concentration of succinate in most tissues. We consider the first reason irrelevant as mechanistic explanations of an empirical fact could be many and changing with advancing of the knowledge in the field. The second argument deserves an extended consideration. *In vitro*, an oxidation of millimolar concentrations of succinate can easily result in the generation of a supraphysiological level of protonmotive force,[37] which would result in artificially high membrane potential in mitochondria. As the latter strongly

affects the rate of ROS production (discussed below), it also would be artificially high, exaggerating the rates of ROS production occurring in cells.[38] Indeed, the concentration of succinate in most tissues is believed to be low, in the 0.2–0.5 mM range, much less than 2–5 mM typically used in experiments with isolated mitochondria. However, succinate concentration increases several fold to the millimolar range in ischemic and hypoxic tissue.[39–41] Even under normal conditions, succinate concentration exhibits several-fold fluctuations from 0.3 to 1 mM in perfused heart.42 Succinate also accumulates in muscle tissue during exercise.[43] Figure 3A shows that ROS production by mitochondria incubated under nonphosphorylating conditions was strongly enhanced even by a twofold to threefold increase in succinate concentration.[44] More recent study demonstrated that succinate strongly enhances mitochondrial ROS emission even in the presence of NAD-linked substrates, which is a more physiological situation.[45] Therefore, dismissing succinate-supported ROS generation as unphysiological is unreasonable. Insofar as intermittent hypoxia is a physiological feature in many tissues, accumulation of succinate during the hypoxic period might also contribute to an increased mitochondrial ROS generation upon reoxygenation.

## A Digression: Nutraceutics As a Way to Control Mitochondrial ROS Production

Another very interesting feature of succinate-supported ROS production is its sensitivity to inhibition with another physiological TCA metabolite. Earlier we demonstrated that malate caused a dose-dependent inhibition of ROS production in nonphosphorylating brain mitochondria oxidizing succinate (Fig. 3B, from Ref. 46). Recently, this finding was confirmed by Muller *et al.*[47] who hypothesized that malate effect can be explained by the oxaloacetate-induced inhibition of succinate dehydrogenase, which is a well-known mechanism where malate in the mitochondrial matrix serves as a precursor for oxaloacetate, which in turn strongly inhibits[48,49] succinate dehydrogenase. The fact that one metabolite can significantly suppress mitochondrial ROS production caused by another metabolite is remarkable. In our opinion, it lends strong support to the validity of “nutraceutics” as an approach to manipulate mitochondrial ROS production *in vivo* in a whole organism. Nutraceutics as an approach to improve mitochondrial functioning is well commercialized in the form of various food supplements, such as vitamins and antioxidants, to be ingested to maintain the health of one’s mitochondria. There is a strong scientific basis to back up this approach.[50] We believe that it may also be possible to control the mitochondrial ROS production by simply selecting food sources rich in certain mitochondrial TCA substrates, such as malate. While this approach awaits its rigorous experimental assessment, it should be noted that there is at least one pioneering example indirectly supporting its validity. In 2001, Prof. John P. Blass reported that giving a mixture containing malate to Alzheimer’s disease sufferers resulted in a noticeable improvement of their mental functions (Challenging Views of Alzheimer’s Disease Conference Cincinnati, Ohio, July 27–29, 2001). To note, mitochondria-associated oxidative stress is thought to significantly contribute to neurodegeneration in Alzheimer’s disease.[51–54]

## Back to the Metabolic Factors Affecting Mitochondrial ROS Production

The magnitude of the membrane potential in mitochondria reflects their metabolic workload. When mitochondria are actively phosphorylating ADP, their membrane potential is approximately 15–20% lower than that in the resting state when no phosphorylation occurs. Other energy-dissipating reactions, such as $Ca^{2+}$ uptake, may induce even stronger decline in the membrane potential. The rate of ROS emission is strongly controlled by the magnitude of the membrane potential. With NAD-linked oxidative substrates, a decrease in the membrane potential associated with an onset of the active phosphorylation results in approximately 30% decrease in the ROS emission rate; a similar decrease in the membrane potential inhibits ROS emission by 90% in mitochondria oxidizing succinate.18 The membrane potential reflects and affects the redox potential of the electron carriers of the respiratory chain. In the absence of respiratory chain inhibitors, the lower the potential, the lower is the reduction level of electron

carriers; it is therefore not surprising that ROS production is decreased at lower membrane potential (see Refs. 18, 33 for a more detailed discussion).

Another metabolic factor known to control the rate of ROS emission is the acidity of the medium. It is well established that mitochondrial ROS emission is inhibited at acidic pH and stimulated at alkaline pH;[55–57] however, there is yet no consensus on the mechanism of this effect.

## Oxygen Tension and Mitochondrial ROS

The effect of the oxygen tension on the rate of ROS production has long been unclear. Whereas earlier work by Britton Chance's group[58] demonstrated that mitochondrial ROS production is enhanced under hyperoxic conditions, the effect of the physiological oxygen concentration had not been explored. It has long been known that tissue oxygen tension is much less than that in ambient air and that the oxygen gradient can be readily observed at the intracellular level.59 It was suggested that, at low intracellular levels of oxygen, mitochondrial ROS production should decline dramatically, whereas the amount of oxygen would still be enough to saturate cytochrome oxidase and maintain normal mitochondrial respiration.60,61 However, recent experiments by Paul Brookes' group do not support this hypothesis.[62] These authors found that ROS emission was almost unaffected by changes in oxygen tension ranging from 250 μM (approximately ambient oxygen) to as low as approximately 5–7 μM (intracellular range). Moreover, the apparent affinity of ROS-producing sites toward oxygen was actually much higher than that of cytochrome oxidase, so the ROS emission remained steady at the low levels of oxygen when the respiration rate was becoming inhibited (Fig. 4, the graph is rederived from the data reported in Ref. 62).

An important aspect of these findings is that it provides a strong argument against the role of mitochondria in so-called hypoxic increase in ROS production. This phenomenon was reported by many groups as a moderate increase in a steady-state level of intracellular ROS occurring at low oxygen tension as compared to normoxic levels of ROS. A dominating explanation of this phenomenon implicated an increase in mitochondrial ROS emission at low oxygen tensions because of some real and perceived properties of the mitochondrial organization that would be irrelevant to discuss here (see Ref. [62] for the references and detailed discussion). However, no increase in ROS emission has been observed with isolated mitochondria at any level of oxygen tension (Fig. 4), meaning that the hypoxic increase in ROS production cannot be attributed to mitochondria per se.[62]

Regarding the hypoxic increase in ROS production, we would like to note that it might be an experimental artifact. Most if not all authors reporting this effect made use of $H_2$DCF-DA fluorescence indicator to detect the levels of intracellular ROS. This molecule can be oxidized, albeit slowly, by various ROS into a fluorescent form, fluorescein, thereby reflecting intracellular ROS levels. However, the reaction of $H_2$DCF oxidation by the superoxide and $H_2O_2$ is complex and unspecific toward these ROS; it is known to be affected by various intracellular factors that are very difficult (if at all possible) to control experimentally (see an excellent recent review[63] by Peter Wardman for a thorough discussion of pitfalls and advantages of this and other ROS probes). Moreover, $H_2$DCF oxidation is strongly enhanced by various hemoproteins and by the reduced cytochrome *c*.[64–66] Therefore, it is not a suitable ROS indicator under conditions when significant changes in the reduction of cytochrome *c* are expected, such as hypoxia. Another example of a seemingly paradoxical enhancement of an oxygen-requiring reaction in hypoxia comes from classical studies on the liver toxicity of $CCl_4$ and several other organic compounds. The toxicity of these compounds is attributed to the lipid peroxidation enhancement by their carbon radicals. A propagation of lipid peroxidation requires oxygen; however the toxicity of $CCl_4$ peaks at some hypoxic levels of oxygen. This had been explained by the fact that $CCl_4$ is activated to its radical form in a

reaction with microsomal cytochrome P450 at a site where $O_2$ usually becomes activated during the monooxygenase cycle of the enzyme. This results in a competition between xenobiotics and oxygen for electrons and a higher rate of radical production at lower oxygen tension.[67] The reactivity of the fluorescent ROS indicators toward similar cellular enzymes is generally not known. Therefore, any results demonstrating an enhancement of ROS production in hypoxia should be considered with caution when fluorescent indicators are involved.

Although the rate of ROS production is apparently not controlled by the oxygen availability in its physiological range, the oxygen is still being consumed to produce ROS and the electrons for the ROS production are derived from oxidative substrates. It is therefore interesting to evaluate how much of the total oxygen consumed by mitochondria is diverted toward ROS production. An earlier and frequently cited evaluation is that about 1–2% of the total consumed oxygen is diverted to ROS production.[58] It is frequently misinterpreted as "1–2% of oxygen consumed by cells or tissue is converted to ROS." It however means that 1–2% of total oxygen consumed by isolated pigeon heart mitochondria in resting state is diverted to the production of $H_2O_2$. This rate is expected to be different in mitochondria from other tissues and species and to depend on the experimental conditions. For example, Table 2 lists the rates of ROS production in percent of the total oxygen consumption by isolated rat brain mitochondria under two major metabolic conditions, the resting and the phosphorylating respiration. A percentage of oxygen diverted to ROS production depends upon both the metabolic condition and the oxidative substrate. It may be safely assumed that an amount of the total electron (and oxygen) flux diverted to ROS formation in mitochondria is tissue and species specific simply because the expression levels of the ROS–producing enzymes exhibit such specificity.[18,68,69]

## Properties of Mitochondrial RDS

Although the presence of many RDS enzymes in mitochondria has long been acknowledged, the capacity and the regulation of RDS in its integrity are little studied. Mammalian mitochondria are well equipped to scavenge the superoxide generated either internally or externally.

The manganese superoxide dismutase (MnSOD, SOD2) is located in the mitochondrial matrix where it protects mitochondrial targets against the internally generated superoxide (reviewed in Ref. 18,70).

At least two other mitochondrial enzymes are capable of efficient scavenging of the externally generated superoxide, CuZn superoxide dismutase (SOD1) and cytochrome *c* plus cytochrome *c* oxidase system (C-COX, Fig. 2). Although most of SOD1 is found in cytosol, some amount of this enzyme can be found in the space between the outer and the inner mitochondrial membranes[71–73] where it can participate in the removal of the superoxide generated outside mitochondria, at least in theory. However, its contribution to the superoxide-scavenging capacity of mitochondria has not been thoroughly investigated. Inarrea *et al.* reported that SOD1 in intact rat liver mitochondria did not participate in the removal of the exogenous superoxide unless the mitochondrial outer membrane was disrupted selectively by digitonin.[72] To note, intact rat liver mitochondria neither scavenged nor dismutated externally generated superoxide at all.[72] This is at variance with other reports documenting quite prominent scavenging of externally produced superoxide by heart,[74,75] liver,[76] and yeast[76] mitochondria.

The second system capable of efficient superoxide scavenging is cytochrome *c* plus cytochrome *c* oxidase. The intermembrane space of mitochondria contains approximately 0.7 mM cytochrome *c*,[77] which can be alternatively reduced by the respiratory chain or superoxide[78] and regenerated (oxidized) by cytochrome *c* oxidase. The oxidation of superoxide-reduced cytochrome *c* by cytochrome *c* oxidase generates proton-motive force that mitochondria can use to produce ATP.[75] The antioxidant properties of cytochrome *c* were

confirmed in experiments with isolated mitochondria,[74] but the physiological role and *in vivo* efficiency of this RDS remain to be explored.

Some known properties of RDS will be discussedhereonthe example of $H_2O_2$ removal by rodent brain mitochondria. It should be clearly understood that these properties of RDS may not be the same in mitochondria from other tissues and species as the expression levels of the RDS enzymes are tissue and species specific.[68,69]

A recent study by Alexandre's group demonstrated that rat brain mitochondria remove $H_2O_2$ at a very high rate of approximately 0.3–6.7 nmol/min/mg protein.[79] The rate of $H_2O_2$ removal exhibited complex dependence on the metabolic state of mitochondria and the nature of oxidative substrate used; the lowest rate (0.3 nmol/min/mg) was observed with deenergized mitochondria incubated in the absence of exogenous substrates. The highest rate of $H_2O_2$ removal (6.7 nmol/min/mg) was observed in mitochondria energized by NAD-linked substrates glutamate and malate incubated in the presence of $Ca^{2+}$ chelator ethyleneglycolbis (β-aminoethyl ether)-N,N'-tetra-acetic acid (EGTA). It should be noted that the highest rate of $H_2O_2$ removal is approximately two to three times higher than a maximum rate of $H_2O_2$ production typically reported with isolated rodent brain mitochondria (e.g., Fig. 2). Authors concluded that $H_2O_2$ removal in brain mitochondria is negatively controlled by $Ca^{2+}$ via inhibition of the glutathione reductase/glutathione peroxidase (GR/GPx) system and that brain mitochondria function as intracellular $Ca^{2+}$-modulated peroxide sinks. Another conclusion that can be drawn from this study is that mitochondrial GR and GPx are the major players in removing exogenous $H_2O_2$.[79]

To note, the rate of $H_2O_2$ removal in experiments by Alexandre's group was measured by following the disappearance of a rather high amount of added $H_2O_2$ bolus (8 nmol in 1.6 ml, 5 μM $H_2O_2$ concentration). The highest reported level of $H_2O_2$ in brain stands as approximately 100 μM as measured by microdialysis in rat striatum after ischemia/reperfusion.[80] However, the steady state levels of $H_2O_2$ in naïve rat brain yielded much lower values, approximately 0.008 μM of $H_2O_2$,[81] whereas typical steady-state $H_2O_2$ concentrations in other tissues and cell cultures have been reported to be in the 0.01–0.1 μM range.[82,83] It would therefore be of interest to assess the ability of mitochondria to remove $H_2O_2$ at these low physiological concentrations. We did such experiments using 0.5–0.8 nmol $H_2O_2$ introduced as a bolus; we also used a $H_2O_2$ generation system comprised of xanthine + xanthine oxidase co-incubated with isolated brain mitochondria and adjusted to generate $H_2O_2$ at 0.005–0.07 nmol/min, which is within a range of $H_2O_2$ emission characteristic for experiments with isolated mitochondria. Our preliminary data agree well with those reported[79] in that the rate of $H_2O_2$ removal is affected by the nature of oxidative substrates and the metabolic state of mitochondria. We also found two important features of RDS that have not yet been reported.

First, brain mitochondria isolated from GPx1 knockout mice devoid of glutathione peroxidase activity[84,85] exhibited exactly the same rates of $H_2O_2$ removal as wild-type mouse brain mitochondria. Thus, it appears that GPx1 is not essential in removing low concentrations of $H_2O_2$. To note, these results should be interpreted with caution as the GPx1 knockout mice might harbor compensatory increases in other $H_2O_2$ scavenging mechanisms thereby obscuring the role of the GPx1 per se.

Second, we found that when $H_2O_2$ was generated by xanthine + xanthine oxidase, there was no continuous accumulation of $H_2O_2$ in the incubation medium. A few tens of seconds after an initiation of $H_2O_2$ generation, its level in the incubation medium reached a steady state and did not change through the duration of the experiment for over 20 min. As anticipated, the steady-state level of $H_2O_2$ was proportional to the rate of $H_2O_2$ production and the content of mitochondria in the medium. Moreover, it was also affected by the nature of oxidative substrate

present in the incubation medium, with succinate producing the highest level and NAD-linked substrates the lowest steady-state levels of $H_2O_2$. Therefore, mitochondria essentially "clamp" or buffer the steady-state levels of $H_2O_2$ (A.A. Starkov *et al.*, manuscript in preparation).

These experiments explain a puzzling earlier observation made by us and by other researchers[86] and discussed in Ref. [18]. When intact mitochondria were co-incubated with a $H_2O_2$ detection system comprising horseradish peroxidase and its fluorogenic substrate, the rate of ROS emission was readily detectable. However, when the $H_2O_2$ detection mix was added to the incubation medium after removing mitochondria, very little of accumulated $H_2O_2$ was detected, grossly disproportional to the expected levels calculated from ROS emission rates measured in the presence of $H_2O_2$ detection mix. A time-dependent accumulation of $H_2O_2$ was observed only when mitochondria were structurally compromised. [18] When succinate was used as an oxidative substrate, an accumulation of $H_2O_2$ was also observed but it was not proportional to the time of incubation prior to removal of mitochondria and adding the $H_2O_2$ detection system (A.A. Starkov *et al.*, manuscript in preparation). The latter is not surprising considering that mitochondria clamp the steady-state level of $H_2O_2$ at a value proportional to the rate of its generation and that succinate supports the highest rate of $H_2O_2$ production in intact brain mitochondria (Fig. 2). On the other hand, structural damage to mitochondria renders their RDS inefficient because of the leakage of GSH and NADPH from the matrix, thereby allowing time-dependent accumulation of $H_2O_2$.

Summarizing, it appears that at least brain mitochondria function as a sink and a source of $H_2O_2$ simultaneously. Their RDS is extremely efficient at higher concentrations of $H_2O_2$, which makes mitochondria a *bona fide sink* of $H_2O_2$. However, RDS is much less efficient at low physiological concentrations of$H_2O_2$,and because of that it buffers the steady state level of $H_2O_2$ at a value proportional to its generation rate and the mass and the metabolic state of mitochondria.

## Role of Mitochondria in Cellular ROS Metabolism and Signaling

Although mitochondria are frequently named the major source of intracellular ROS in recent literature, they are definitely not the only source. Numerous other cellular sources of ROS have been reported, for example, enzymes, such as membrane-bound NADH and NADPH oxidases, xanthine oxidase, eNOS, lipoxygenases, cyclooxygenases, microsomal metabolism of xenobiotics, and peroxisomal β-oxidation of fatty acid (reviewed in Ref. [14] and numerous reviews cited therein). Although many of these sources are capable of substantial ROS production, the latter is either limited by the availability of their substrates or requires a specific activation by other factors. In other words, *the ROS generation by nonmitochondrial sources exhibit spatial and temporal discontinuity pertinent to metabolic fluctuations in a particular cell in a particular moment of time*. On the other hand, mitochondria are ubiquitously present in cells and generate ROS continuously as long as they have access to oxygen and oxidative substrates. However, the amount and protein composition of mitochondria vary among tissues and the level of ROS they generate also reflects metabolic fluctuations. Moreover, many of nonmitochondrial ROS-producing enzymes are also ubiquitously expressed and may contribute to the ROS production in parallel with mitochondria. Currently, it is not technically possible to systematically compare their ROS-producing capacity with that of mitochondria under realistic metabolic conditions. Multiple sources of ROS in mitochondria co-exist with a powerful multileveled RDS system. The contribution of each of those systems in the overall cellular ROS metabolism is expected to exhibit the species and tissue specificity and therefore it has to be determined on a case-by-case basis. Questions, such as "are mitochondria a major source/sink/target of ROS in cells" could not be answered in general.

Overall, it may be suggested that mitochondria serve as an important ROS-stabilizing feedback node in the cellular ROS-signaling network. Being a source and a sink of ROS at the same time, they "sense" changes in the intracellular metabolic demands, nutrition supply, their own abundance and functional and structural integrity, and the external ROS "pressure," and "report" the integrated signal back to the cell in the form of the steady-state level of $H_2O_2$ set forth by a balance between their $H_2O_2$ emission and removal rates (Fig. 5).

## Conclusion

Irrespectively of whether or not mitochondria are the major source of ROS in cells, strategies toward diminishing their contribution to intracellular oxidative stress are of great practical interest. However, considering various strategies aiming to decrease mitochondrial ROS, there is one important question to be answered. This question is "what exactly should be decreased?" Indeed, one should distinguish between the generated ROS, the emitted ROS, and the scavenged ROS. The *generated ROS* is an amount of ROS primarily produced in mitochondrial sites. Insofar as we can deduce from *in vitro* experiments with isolated mitochondria (which contributed approximately 99% of all that we know about mitochondrial functions), it should be possible to decrease this "innate" ROS generation by either changing a content/properties of the ROS-generating sites genetically or by targeting mitochondrial metabolism through nutraceutical or pharmacological approaches. While the former approach appears to be rather dubious (at this time), the second approach seems to be workable, to some extent, through metabolic interventions, such as caloric restriction or changing an intracellular pattern of mitochondrial oxidative substrates through nutrition supply. The *emitted ROS* is a difference between the generated ROS and the ROS scavenged by the mitochondrial RDS. Theoretically, it should be possible to change the emission of ROS by either manipulating the ROS generation or by changing the affinity of ROS-scavenging reactions in RDS to the mitochondria-generated ROS. However, the physiological level of ROS emission from mitochondria is negligible (as discussed in this review) and unlikely to be of any significance except as a signal. Therefore, it is not clear whether diminishing the ROS emission below its physiological level would be beneficial. It might, of course, be desirable in a pathology associated with a severe oxidative stress caused by a supraphysiological ROS production from damage to mitochondria.

Alternatively, increasing the capacity of mitochondrial RDS toward the faster removal of supraphysiological levels of ROS seems quite attractive under all circumstances. It might be achieved by enhancing the mitochondrial RDS with nonenzymatic antioxidants[87] and metabolites supplying the reducing equivalents to RDS (e.g., malate, isocitrate), or by a selective stimulation of expression of RDS components. Taking into account the properties of mitochondrial RDS as discussed above, it seems unlikely that increasing its capacity toward removal of high levels of ROS would change the physiological ROS signaling mediated by the ROS emission from mitochondria. However, it would provide a better guard against any abrupt increase in the steady-state ROS levels caused by extracellular or intracellular events.

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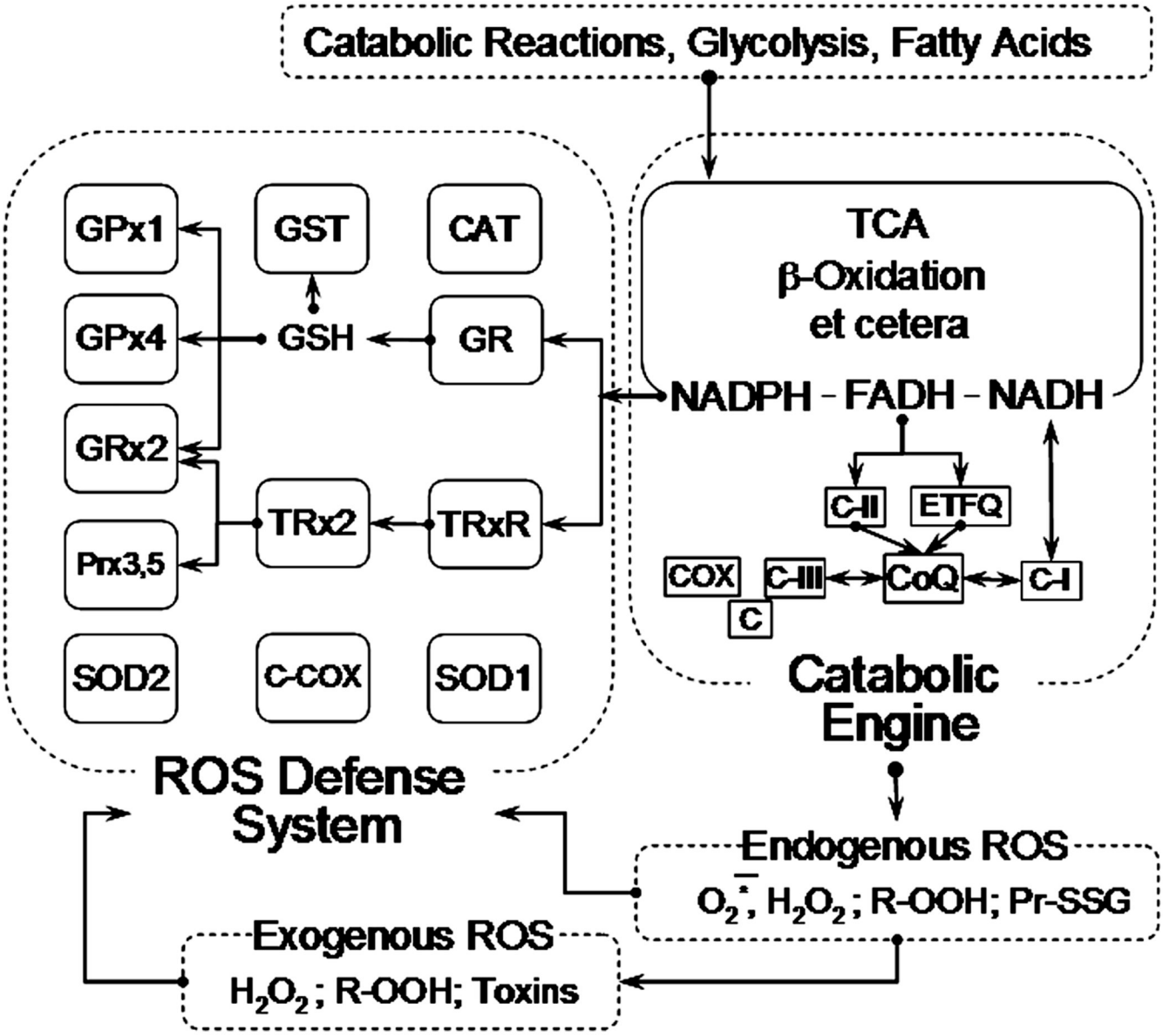


**Figure 1.**

Mitochondrial catabolic engine and reactive oxygen species (ROS) defense system. See text and Ref. [18] for further detail. Abbreviations: GPx1, mitochondrial glutathione peroxidase; GPx4, mitochondrial phospholipid hydroperoxide glutathione peroxidase; Grx2, glutaredoxin-2; Prx3,5, peroxiredoxins 3 and 5; SOD2, mitochondrial manganese superoxide dismutase; GST, glutathione-S-transferase; GSH, reduced glutathione; TRx2, thioredoxin-2; CCOX, cytochrome *c* plus cytochrome *c* oxidase; Cat, catalase; GR, gluthatione reductase; TRxR, mitochondrial thioredoxin-2 reductase; SOD1, CuZn superoxide dismutase; TCA, tricarboxylic acid cycle; C-II, succinate dehydrogenase; ETFQ, electron transferring flavoprotein:CoQ reductase; CoQ, coenzyme Q; CI, Complex I of the respiratory chain; C-III, Complex III of the respiratory chain; C, cytochrome *c*; COX, cytochrome *c* oxidase.

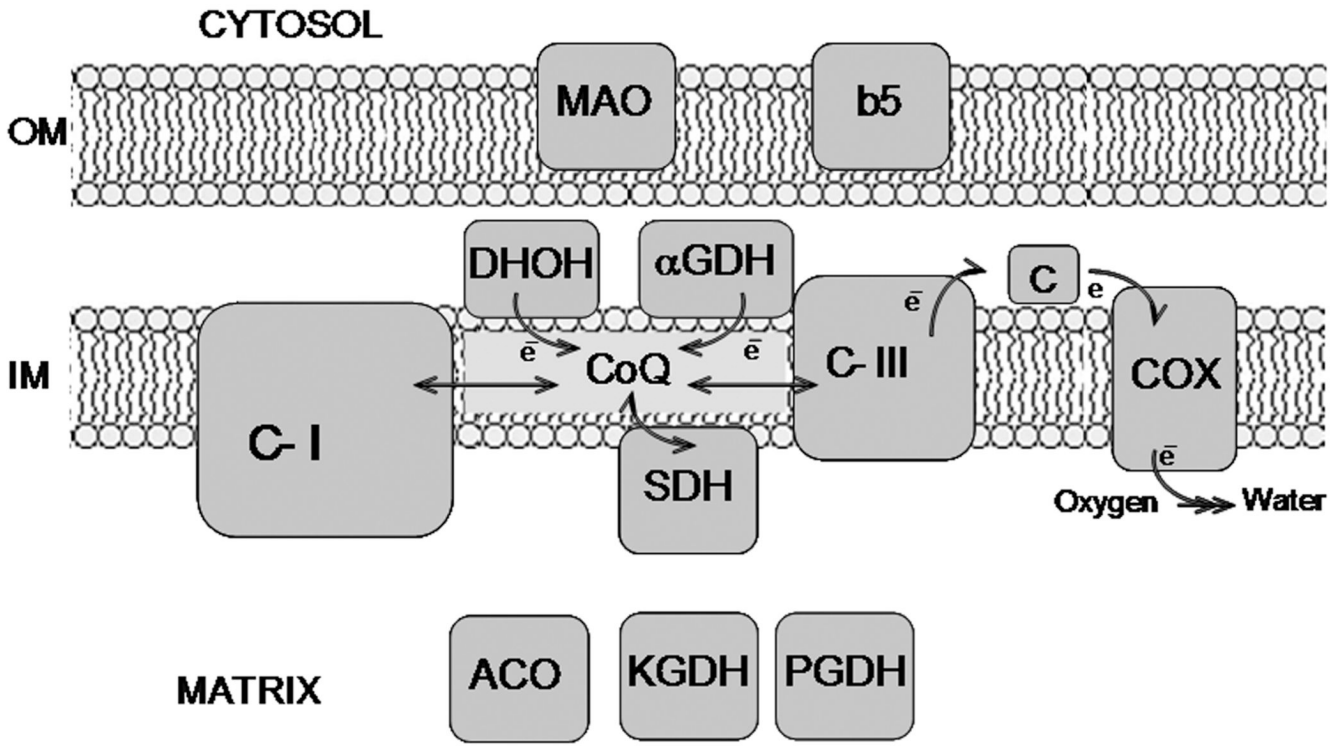


**Figure 2.**
Reported mitochondrial sites capable of ROS generation. Known ROS-generating enzymes are shown in a context of their location within mitochondria. See text and Ref. [18] for further detail. Abbreviations: OM, outer mitochondrial membrane; IM, inner mitochondrial membrane; MAO, mono amine oxidases A and B; b5, cytochrome b5 reductase; DHOH, dihydroorotate dehydrogenase; αGDH, α-glycerophosphate dehydrogenase; C-I, Complex I of the respiratory chain; CoQ, coenzyme Q; C-III, Complex III of the respiratory chain; C, cytochrome *c*; COX, cytochrome *c* oxidase; SDH, succinate dehydrogenase; ACO, aconitase; KGDHC, α-ketoglutarate dehydrogenase complex; PDHC, pyruvate dehydrogenase complex; e, electrons. Arrows indicate the direction of electron flux between the enzymes and CoQ.

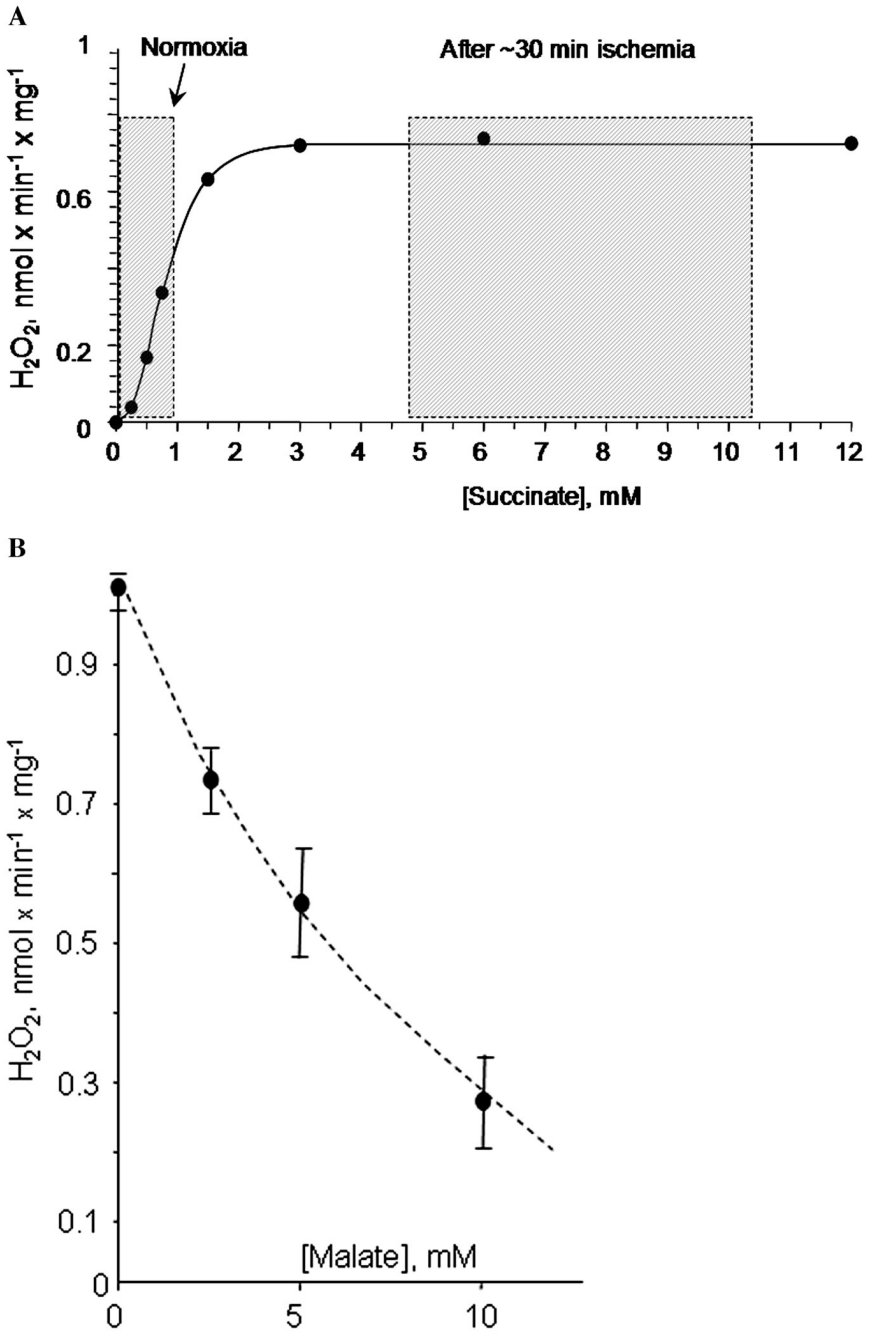


**Figure 3.**

Features of succinate-supported ROS emission in rat brain mitochondria. **(A)** Concentration dependence of succinate-supported ROS emission in rat brain mitochondria incubated under nonphosphorylating conditions. Shaded boxes indicate the range of tissue succinate concentrations typically observed under normoxic conditions (normoxia) and after 20–30 min of hypoxia ischemia. See text for further detail. **(B)** Malate inhibits succinate-supported ROS emission in rat brain mitochondria incubated under nonphosphorylating conditions. Experimental conditions: incubation buffer was composed of 125 mM KCl, 2 mM $KH_2PO_4$, 1 mM $MgCl_2$, 0.2 mg/ml of fatty acids-free BSA, 20 mM HEPES (pH 7.2), 0.2 mM EGTA, and **(A)** pictured concentrations of succinate or **(B)** 5 mM succinate. Mitochondria were added

at 0.25 mg/ml; $t = 37°$. The emission of $H_2O_2$ was estimated from the changes in fluorescence using an $H_2O_2$ detection mixture of 4 U/ml of horseradish peroxidase, 40 U/ml SOD, and 10 μM Amplex Red (Molecular Probes, Oregon, USA).

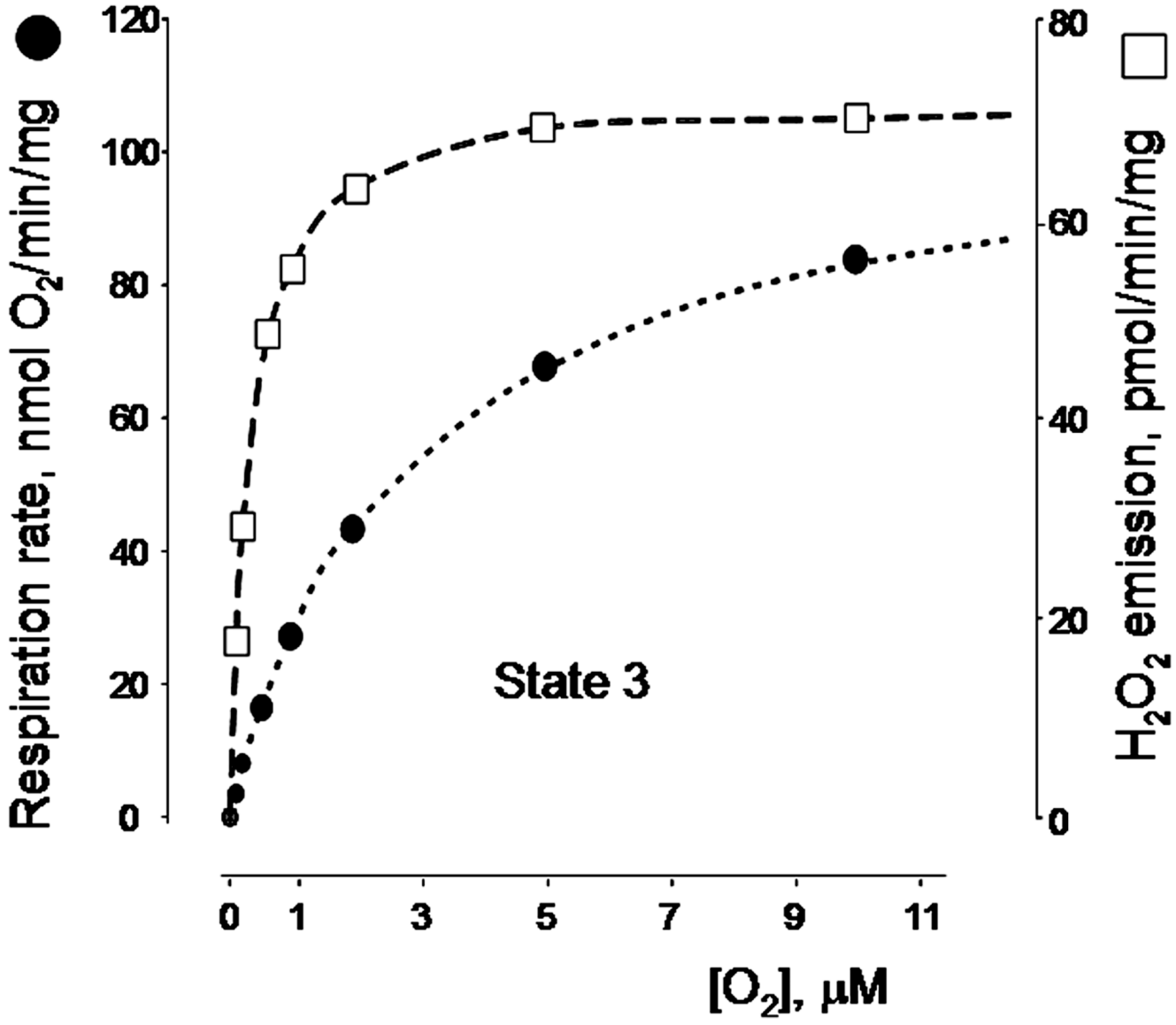


**Figure 4.**
Effect of the oxygen tension on the rates of phosphorylating respiration and $H_2O_2$ emission in rat liver mitochondria. The graph was rederived from the data reported in Ref. [62]. See text for the detail.

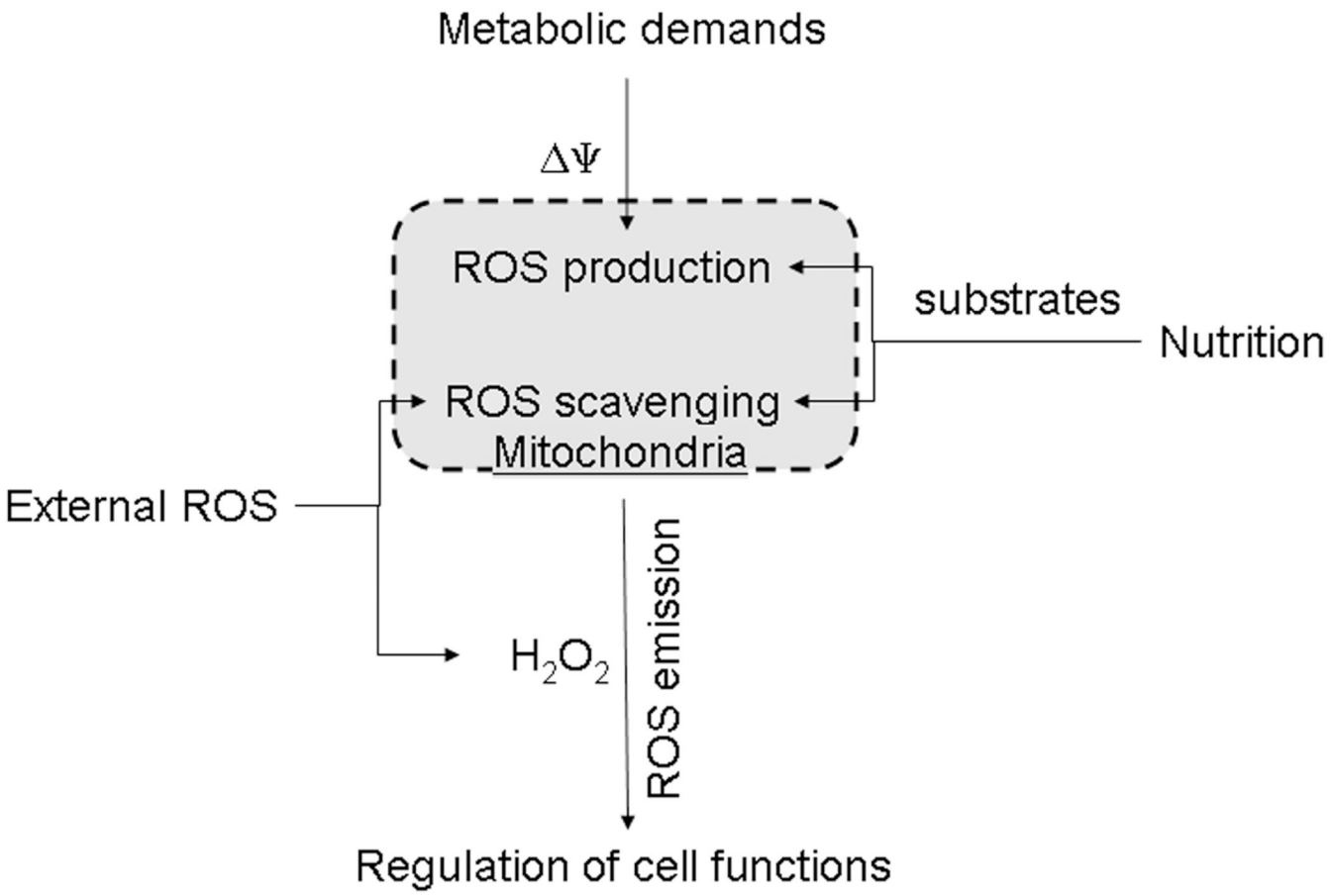


**Figure 5.**
Mitochondria sense changes in metabolism and ROS pressure and respond with an integrated signal in the form of $H_2O_2$. The magnitude of the mitochondrial membrane potential ($\Delta\Psi$) changes following fluctuations in the demands, such as ATP synthesis, which are imposed by cellular metabolism on the mitochondrial catabolic engine. Changes in cell nutrition are reflected by changes in the pattern of available oxidative substrates, monoamine oxidase substrates, and other factors. This results in changes in ROS generation in various mitochondrial sites. Internally generated ROS are combined with extramitochondrial ROS and get filtered through the mitochondrial ROS-scavenging system. The resulting level of the $H_2O_2$ emission serves as an integrated feedback signal from mitochondria to other intracellular $H_2O_2$-sensing systems.

## TABLE 1

A Comparison of the Reactive Oxygen Species (ROS)-Generating Capacities in the Various Mitochondrial Sites

| ROS-producing site | $H_2O_2$ generation range, nmol/min/mg | Conditions |
|---|---|---|
| RET | 1.0–3.0 | Succinate or α-glycerophosphate, State 4 respiration |
| FET | 0.06–0.4 | glutamate + malate, State 4 respiration |
| Max C-I + Matrix Dehydrogenases | 0.1–0.4 | NAD-linked substrates + rotenone |
| C-I | 0.3–0.6 | NADH + rotenone, permeabilized mitochondria |
| C-III | 0–0.2 | Succinate + rotenone + antimycin A, intact mitochondria |
| Max C-III | 0–2.0 | Succinate + fumarate + antimycin A, permeabilized mitochondria |
| MAO | 0.7–1.5 | 100 μM Kynurenin, no oxidative substrates |

This table presents a compilation of data obtained by the author of this review in numerous (done over about a 5-year period) experiments with isolated rat brain mitochondria (except for the MAO data, which were obtained with isolated nonsynaptic mouse brain mitochondria, C57/Bl6 strain, $n = 6$).

Abbreviations: RET, reverse electron transfer, represents $H_2O_2$ generation by isolated rat brain mitochondria incubated under conditions described in Figure 3. Note that although Complex I is frequently considered as the site of ROS generation under these conditions, this concept has no stringent experimental support because ROS generation in other NAD-linked intramitochondrial sites, such as dihydrolipoamide dehydrogenase cannot be excluded (discussed in Ref. 18); FET, forward electron transfer, represents $H_2O_2$ generation supported by the oxidation of NAD-linked substrates; State 4 describes a nonphosphorylating resting respiration state when all the energy expenditures experienced by mitochondria originate from an inherent proton leak through the inner mitochondrial membrane; max C-I + matrix dehydrogenases is $H_2O_2$ emission measured with isolated mitochondria supplied with NAD-linked oxidative substrates in the presence of Complex I (C-I) inhibitor, rotenone, which blocks the net-electron transfer through the respiratory chain. As in the case of FET, an involvement of various NAD-linked enzymes located in the mitochondrial matrix in the generation of ROS cannot be excluded under these conditions; C-I, mitochondria were permeabilized with a pore-forming peptide, alamethicin, washed out of low-molecular weight matrix components, and supplemented with 100 μM NADH; max C-III corresponds to the maximum generation of ROS at the level of Complex III of the respiratory chain. See Ref. [86] for details.

## TABLE 2

Substrate-Specific Mitochondrial ROS Emission Expressed in Percent to the Rate of Oxygen Consumption

| | $H_2O_2$ production (% from the respiration rate) | |
|---|---|---|
| **Substrate** | **Resting respiration** | **Phosphorylating respiration** |
| Citrate | 0.71 | 0.19 |
| Glutamate | 0.33 | 0.05 |
| Malate | 0.76 | 0.07 |
| Malate + Glutamate | 0.79 | 0.08 |
| α-Ketoglutarate | 1.07 | 0.07 |
| Pyruvate | 0.89 | 0.19 |
| Succinate | 3.15 | 0.04 |

Experimental conditions were as described in Figure 3. The phosphorylating respiration rates were recorded under the same conditions except that the incubation buffer was supplemented with 400 μM ADP. All substrates were added at 5 mM concentration except for the malate plus glutamate, which were added at 1 and 5 mM, respectively.