1 Mitochondrial ROS Production

Anatoly Starkov and Kendall B. Wallace

1. Introduction

For many, it is a firm paradigm that mitochondria are the major source of reactive oxygen species (ROS) in mammalian cells; dissenters consider these complicated organelles the major target of oxidative stress, whereas conformists argue that mitochondria are both the source and target of intracellular ROS, subject to conditions. All these views are possibly correct because the reality is that there is not enough data yet to support any scientifically based conclusion on the role of mitochondria in the intracellular ROS metabolism. Being able to produce ROS at least in vitro, mammalian mitochondria also possess powerful, multi-leveled high-capacity ROS defense systems that are not well studied. It is not yet understood what function — ROS production or ROS scavenging — prevails in mitochondria in vivo. This review attempts to introduce major elements of both ROS producing and detoxifying systems mitochondria encompassing the state of the art circa 2004. Summarized are the major findings regarding the mitochondrial sites of ROS production, the regulation of ROS production, and the ROS defense systems relevant primarily to the mammalian mitochondria.

2. Multiplicity of ROS-Producing Sources in Mitochondria

Although about ~50% of all the land in Holland lies below sea level, the assiduous and diligent Hollanders created numerous dikes and channels and other things to hold the sea back so that the land can be put to good
A familiar fable tells the story that long ago, in a city named Haarlem, there lived a boy named Peter who was just eight years old, but he was very smart and swift-minded. One rainy day as he walked home after a visit to the countryside all by himself, he suddenly heard a sound of water springing from a small hole in the dike. Peter examined that situation and quickly calculated that the pressure of the water would make the hole huge pretty soon causing the dike to burst in a wall of water bringing flood and disaster to the town below! He immediately saw a solution; Peter got onto the dike and stuck his finger in the hole. It was cold and dark and he started feeling quite miserable. He knew no help would come to his rescue soon but decided rather to die holding the water back than betray his beautiful country. There he was, an eight-year-old boy lying on his tummy on a cold damp dike holding the flood with his finger all through the night. When dawn broke, a cleric walked by and saw Peter, and inquired about what was going on. Peter’s answer was simple: “I am holding the water back,” he said, “Please get help!” And help he got, the compassionate cleric climbed up to his side and put his palm over the boy’s small hand to prevent his tired finger from falling out of the hole in the dike. Haarlem was saved and the boy has been revered as Holland’s national hero ever since.

Hydraulic analogy with a dike and a hole illustrates the essence of mitochondrial ROS production. A source of ROS is like a hole in the dike; it brings more damage the longer it is left unpatched, and eventually destroys the “city of Mitochondrion.” The major difference however is the number of fingers required to prevent a disaster.

Thermodynamically, numerous mitochondrial enzymes and enzyme complexes are capable of one-electron reduction of oxygen. ROS production by at least nine of the mammalian mitochondrial enzymes has so far been reported by various laboratories; it is highly likely that additional ROS sources will be discovered as many more researchers started exploring the field in the last four years than during the last four decades. Although all nine ROS-producing enzymes are more or less ubiquitously present in mammalian mitochondria, their capacity in producing ROS varies greatly and there is always a tissue specificity factor as everything is expressed to different levels in different tissues. Adding to the metabolic heterogeneity of tissues and our limited knowledge of life’s mechanics, it is not surprising
that singling out a ROS-producing source as the major one in vivo might be difficult if not impossible, scientifically speaking.

The nine known sources of ROS in mitochondria (marked by stars) are shown on Fig. 1 in the context of location within a mitochondrion. ROS production by Complex I (C-I) and Complex III (bc1) will be discussed in separate sections, as well as numerous ROS-detoxifying systems presented on Fig. 1. This section introduces the remaining seven ROS-producing enzymes.

(1) Mitochondrial cytochrome b5 reductase is located in the outer mitochondrial membrane. The enzyme is widely distributed in mammalian tissues. It oxidizes cytoplasmic NAD(P)H and reduces cytochrome b5, another protein of the outer membrane. Cytochrome b5 reductase may be involved in regeneration of ascorbate because it catalyzes the reduction of ascorbyl free radical back to ascorbate in mammalian liver and in yeast mitochondria. It may play some important role in human brain cells; it is elevated in schizophrenics thus implying having a role in aetiology of the disease. There is a single report that mitochondrial cytochrome b5 reductase using NADH as an electron donor may produce superoxide with a very high rate ∼300 nmol superoxide per min per mg protein. Few other details or other studies on this subject are currently available.

(2) Monoamine oxidases (MAO-A and MAO-B, EC 1.4.3.4) are also located in the outer mitochondrial membrane and ubiquitously expressed in various mammalian tissues. These enzymes catalyze the oxidation of biogenic amines accompanied by the release of H2O2. MAOs of brain mitochondria play a central role in the turnover of monoamine neurotransmitters; numerous detailed and extensive reviews covering almost every aspect of these enzymes can be found elsewhere. The amount of H2O2 that MAOs can generate may substantially exceed the amount produced by any other mitochondrial source of ROS. Tyramine oxidation by rat brain mitochondria produced H2O2 with a rate ∼50 times higher than that exerted by Complex III inhibited with antimycin A. The latter ROS-producing system has long been considered as one of the most “productive” sources of ROS in mitochondria (discussed later). Mitochondrial MAO enzymes may also be a major source of H2O2 in tissues in ischemia, aging, and upon oxidation of exogenous biogenic amines. An increase in MAO activity and
Fig. 1. Known sources of ROS and ROS-detoxifying systems in mitochondria. Selected ROS-producing enzymes and ROS-detoxifying systems are shown in a context of their location within mitochondria. See text for further detail. Abbreviations: COX, cytochrome c oxidase; C, cytochrome c, C-III, Complex III; MnSOD, mitochondrial manganese superoxide dismutase; Cat, catalase; SDH, succinate dehydrogenase; ACO, aconitase; Prx3_red, peroxiredoxin reduced; Prx3_ox, peroxiredoxin oxidized; Q, coenzyme Q; DHOH, dihydroorotate dehydrogenase; KGDHC, a-ketoglutarate dehydrogenase complex; αGDH, α-glycerophosphate dehydrogenase; PDHC, pyruvate dehydrogenase complex; IDH, isocitric dehydrogenase, NAD⁺-dependent; Trx2_red, thioredoxin-2 reduced; Trx2_ox, thioredoxin-2 oxidized; Grx2_red, glutaredoxin-2 reduced; Grx2_ox, glutaredoxin-2 oxidized; TrxR2, thioredoxin-2 reductase; MDH, malate dehydrogenase; IDH₁, isocitric dehydrogenase, NADP⁺-dependent; ME, malic enzyme NADP⁺-dependent; GR, glutathione reductase; GSH, reduced glutathione; GS-SG, oxidized glutathione dipeptide; GPs, glutathione peroxidase; PGPxs, phospholipid hydroperoxide glutathione peroxidase; C-1, Complex I; TH, transhydrogenase; Cyt. B5 reductase, cytochrome b5 reductase; MAOs, mono amine oxidases A and B; OM, outer mitochondrial membrane; IM, inner mitochondrial membrane. Other symbols: ROS species that are detoxified by the corresponding systems are shown enclosed in a square frame; underscored numbers correspond to the numbers assigned to a ROS–detoxifying system as described in Sec. 5; stars indicate sources of ROS.
MAO-catalyzed H$_2$O$_2$ production may be responsible for the mitochondrial damage in Parkinson’s disease.\textsuperscript{11}

(3) Dihydroorotate dehydrogenase (DHOH, EC1.3.3.1 or EC1.3.99.11) is located at the outer surface of inner mitochondrial membrane. It catalyzes the conversion of dihydroorotate to the pyrimidine base, orotate, which is a step in the \textit{de novo} synthesis of uridine monophosphate. The latter is involved in the formation of DNA and RNA. The DHOH is ubiquitously distributed in mammalian tissues.\textsuperscript{12} In the absence of its natural electron acceptor, coenzyme Q of inner mitochondrial membrane, reduced DHOH can produce H$_2$O$_2$ \textit{in vitro}.\textsuperscript{12} The DHOH has frequently been considered as a mitochondrial source of superoxide.\textsuperscript{13,14} However, in a more recent study the same authors concluded that superoxide production during dihydroorotate oxidation was from Complex III rather than from DHOH\textsuperscript{15} and explained this and other discrepancies by the lower quality of mitochondrial preparation in the earlier study. Therefore, the capacity of DHOH to produce superoxide requires further clarification.

(4) Mitochondrial dehydrogenase of $\alpha$-glycerophosphate (aka Glycerol-3-Phosphate Dehydrogenase, aka mGPDH, EC 1.1.99.5) is also located at the outer surface of inner mitochondrial membrane. It is a FAD-linked enzyme catalyzing the oxidation of glycerol-3-phosphate to dihydroxyacetone phosphate and utilizing mitochondrial coenzyme Q as electron acceptor. The mGPDH is involved in lipid metabolism and in the so-called glycerol phosphate shuttle capable of regenerating cytosolic NAD$^+$ from the NADH formed in glycolysis. Homozygous mice with disrupted mGPDH survive but have decreased viability and lower body weight than their wild type littermates.\textsuperscript{16} The enzyme expression is upregulated in hyperthyroid animals.\textsuperscript{17,18} It is ubiquitously but unevenly expressed in various mouse tissues with brown fat, muscle, and brain possessing the highest activity of mGPDH.\textsuperscript{19} The activity of mGPDH is also high in flying muscles in insects.\textsuperscript{20} Mitochondria from mouse tissues\textsuperscript{21} and from \textit{Drosophila} fly\textsuperscript{22} produce H$_2$O$_2$ upon oxidation of sn-glycerol-3-phosphate, a substrate of mGPDH. The mechanism of mGPDH-mediated ROS production was studied in \textit{Drosophila} mitochondria, it appeared that most of H$_2$O$_2$ was produced by the enzyme \textit{per se} whereas about 30% was produced at Complex I site because of reverse electron transfer from mGPDH to that site\textsuperscript{22} as discussed later in this chapter.
(5) **Succinate dehydrogenase complex** (SDH, *aka* succinate:ubiquinone oxidoreductase, Complex II, EC1.3.5.1) is a flavoprotein located at the inner surface of inner mitochondrial membrane. The enzyme oxidizes succinate to fumarate using coenzyme Q as an electron acceptor. Although oxidation of succinate by good-quality mitochondria from most mammalian tissues can produce ROS with a high rate, the source of ROS is Complex I, not SDH. The mechanism involves reverse electron transfer from SDH-reduced coenzyme Q to Complex I. Nevertheless, isolated SHD reconstructed in liposomes can produce ROS by itself. Authors concluded that reduced FAD of SDH generates ROS in the absence of its electron acceptor. There is also a report implying that SDH can generate ROS in submitochondrial particles. However the conclusion was based solely on the inhibition of ROS production by carboxin, a specific inhibitor of SDH. The same inhibitor also suppressed antimycin-induced ROS production and ROS production supported by NADH oxidation. The former is thought to originate from Complex III (discussed later) that is not inhibited by carboxin whereas the effect of carboxin on NADH-supported ROS production may not be readily explained either. Therefore, it is unclear whether SDH produces ROS in situ, in mitochondria.

(6) **Mitochondrial aconitase** (m-aconitase, EC4.2.1.3) is an enzyme localized to the matrix space of mitochondria; it participates in tricarboxylic acid cycle catalyzing a conversion of citrate to isocitrate. The enzyme contains an iron-sulfur cluster that can be oxidized by superoxide, inactivating m-aconitase. Recently, it was found that isolated aconitase oxidized by either superoxide or hydrogen peroxide produces hydroxyl radical. The authors proposed that similar continuous hydroxyl radical production may occur upon superoxide-driven redox-cycling of aconitase in mitochondria.

(7) **Ketoglutarate dehydrogenase complex** (KGDHC, *aka* 2-oxoglutarate dehydrogenase) is an integral mitochondrial enzyme tightly bound to the inner mitochondrial membrane on the matrix side. In the tricarboxylic acid cycle, it catalyzes the oxidation of α-ketoglutarate to succinyl-CoA using NAD⁺ as electron acceptor. Structurally, KGDHC is composed of multiple copies of three enzymes: α-ketoglutarate dehydrogenase (E1k subunit, EC 1.2.4.2), dihydrolipoamide succinyltransferase (E2k subunit, EC 2.3.1.12), and lipoamide dehydrogenase (E3 subunit, EC 1.6.4.3). The E3
component of KGDHC is a flavin-containing enzyme; it is identical to the E3 component of another integral mitochondrial enzyme located in the matrix, pyruvate dehydrogenase (PDHC). The E3 component is also known as dihydrolipoamide dehydrogenase (Dld) which is ubiquitously present in mammalian mitochondria. Two recent studies demonstrated that both PDHC and KGDHC can generate superoxide and hydrogen peroxide; ROS production was shown with isolated purified enzymes from bovine heart\textsuperscript{28,29} and in isolated mitochondria.\textsuperscript{28} The source of ROS in KGDHC appears to be the dihydrolipoamide dehydrogenase component.\textsuperscript{28} Earlier, isolated dihydrolipoamide dehydrogenase was shown to produce ROS.\textsuperscript{30} In mitochondria and with isolated enzyme, ROS production from KGDHC was stimulated by a decrease in availability of its natural electron acceptor, NAD\textsuperscript{+}.\textsuperscript{28,29}

To summarize, we would like to emphasize that although these seven sources were shown to produce ROS, in experiments with isolated enzymes or in mammalian mitochondria, there is no way to estimate their contribution to ROS production in mitochondria under physiological conditions. That does not mean of course that it can not be done; genetic engineering and biophysical approaches are ripe and quite suitable for such studies.

3. ROS Production at Complex I of Mitochondrial Respiratory Chain

Mitochondrial Complex I, aka “Rotenone-Sensitive Mitochondrial NADH-Ubiquinone Oxidoreductase”, provides a major entry point into respiratory chain for electrons derived from the oxidation of various substrates in the mitochondrial tricarboxylic acid cycle. It is a very important enzyme catalyzing the oxidation of NADH in the mitochondrial matrix by coenzyme Q dissolved in the inner mitochondrial membrane. It utilizes the energy of NADH oxidation to generate a proton gradient between a mitochondrion and its surroundings that is used to synthesize ATP or other work that mitochondria perform. Many scientists insist that in addition to that, Complex I routinely generates significant amounts of ROS.

Several research groups have demonstrated that Complex I preparations can generate ROS\textsuperscript{1,31,32} when reduced with NADH, although there is no consensus about the specific site of ROS production in Complex I.\textsuperscript{32–34} The
published studies differ widely in approaches, sources of mitochondria and techniques employed for Complex I isolation so the lack of consistency in results is not surprising.

A few studies attempted localizing the ROS producing site or sites within Complex I by using inhibitors of electron transfer. One of the earliest studies demonstrated that isolated Complex I supplemented with NADH can generate superoxide. ROS generation apparently required a reduced ubiquinone molecule because it was inhibited by rotenone which blocks electron transfer from electron-carrying components of Complex I to ubiquinone. The same authors also demonstrated an enhancement of ROS production in Complex I by added quinones, which was later confirmed by. An inhibition of ROS production in NADH-reduced Complex I by rotenone is a unique observation; in other studies cited in this chapter rotenone either enhanced ROS production by NADH-reduced Complex I or had no effect.

Studies with both isolated Complex I and submitochondrial particles demonstrated that ROS producing site is located between a rotenone-sensitive site and a flavin and that there may be not one but two superoxide producing sites in that region. Others suggested that the ROS producing site in Complex I is exactly the flavin or a complex of bound half-reduced NAD$^+$ with the flavin of the enzyme. The sum of presently available data favors the idea that ROS is most likely produced by one of the electron-transferring iron-sulfur centers that are localized in Complex I between the flavin and the rotenone–sensitive site, not by a flavin per se. That may of course change as our knowledge of electron transfer mechanics in Complex I becomes more detailed.

At the intact mitochondria level, two major experimental paradigms are employed in studies on ROS production attributed to Complex I. The first, both historically and by the frequency of use, is ROS production resulting from so-called reverse electron transfer in the mitochondrial respiratory chain. Discovered in experiments with submitochondrial particles, it was the first reaction of ROS production in mitochondria studied in detail. Reverse electron transfer (RET) is a term describing a set of redox reactions in the mitochondrial respiratory chain that allows electrons to flow from coenzyme Q to NAD$^+$ instead of oxygen. It is not yet clear whether or not it is a physiologically relevant phenomenon. RET requires a combination
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of several factors to occur simultaneously. In submitochondrial particles, 
it requires the presence of succinate to reduce coenzyme Q, the electron 
flow should be inhibited downstream, either at the level of Complex III 
or at cytochrome c oxidase and an additional source of energy such as 
hydrolyzable ATP should be present to “push” the electrons from coen-
zyme Q against the redox potential difference toward reduction of NAD⁺. 
And of course, submitochondrial particles should be of very good quality 
having no significant impairments to their respiratory chain components or 
the lipid membrane. If all these conditions are met, a succinate and ATP-
dependent reduction of added NAD⁺ can be observed that is associated 
with a massive production of H₂O₂.¹ ³ Both the NAD⁺ reduction and the 
production of H₂O₂ can be prevented by Complex I inhibitors acting at the 
rotenone-binding site, thereby indicating that the site of ROS production is 
located somewhere in the Complex I upstream of that site. Neither NAD⁺ 
per se nor the electron flow to NAD⁺ is required for ROS production; 
however more ROS is produced in the presence of NAD⁺.⁴³

In mitochondria, RET does not require ATP and an inhibition of elec-
tron flow toward oxygen. It requires only the presence of a FADH₂-linked 
substrate to reduce coenzyme Q directly, and the presence of high mem-
brane potential. These conditions are usually met by incubating mitochon-
dria under resting state conditions (so-called “State 4” respiration) in the 
presence of succinate or α-glycerophosphate.²² ³³ ⁴⁴ In mitochondria, RET 
supports very high rates of ROS production. Rodent heart and brain mito-
chondria oxidizing succinate in State 4 conditions can produce H₂O₂ with 
rates ranging from 0.5 to 3 nmols H₂O₂ per minute per mg of mitochondrial 
protein.⁴⁴ ⁴⁷ That amounts to 5–20% of their total oxygen consumption 
rate under State 4 conditions. RET-induced ROS production is regulated 
by the amplitude of mitochondrial electrical membrane potential⁴⁴ ⁴⁷ so 
that a 10% decrease in the membrane potential inhibits ROS production by 
90%. It is therefore inhibited by any energy-dissipating process, whether 
it is ATP synthesis, Ca²⁺ uptake, or a chemical-induced uncoupling. RET-
supported ROS production is also apparently suppressed by acidification 
of the mitochondrial matrix.⁴⁸ This may be viewed as additional indirect 
evidence that it originates from Complex I; it is known that ROS produc-
tion by Complex I in submitochondrial particles is higher at more alkaline
pH.\textsuperscript{38,49} And of course, RET-supported ROS production in intact mitochondria is inhibited by rotenone because it blocks the flow of electrons from coenzyme Q to Complex I.

The second experimental paradigm in research on ROS production by Complex I starts where the first ends. It has long been known that rotenone induces ROS production by mitochondria oxidizing NAD-linked substrates such as pyruvate or glutamate plus malate. Rotenone-induced ROS production is not regulated by the membrane potential, but it depends on pH\textsuperscript{38,49} and also on the degree of reduction of matrix pyridine nucleotides.\textsuperscript{33} Rotenone-induced ROS production rates are generally about 5–10\% of those induced by succinate-supported RET. It is not known if the same or different sites are involved in RET and rotenone-induced ROS production.

Regarding the physiological relevance of these two experimental paradigms, both may be equally meaningful. The essence of RET-paradigm is high membrane potential that is needed to overcome a redox potential difference between coenzyme Q and a site in Complex I that produces ROS. This condition could occur \textit{in vivo} when mitochondria are in a “resting” non-phosphorylating state, or their phosphorylation is inhibited by a toxic compound. The essence of rotenone-induced ROS production is an over-reduction of intrinsic Complex I electron carriers and mitochondrial pyridine nucleotides. This may also occur \textit{in vivo} due to a xenobiotic or a pathology preventing electron transfer from either Complex I to coenzyme Q or at any point downstream the respiratory chain. For example, a release of cytochrome c from mitochondria due to apoptotic stimuli would result in an enhanced ROS production from Complex I.\textsuperscript{33} Switching of mitochondria into State 4 non-phosphorylating conditions also enhances ROS production by mitochondria oxidizing NAD-linked substrates, but to a 10–20 times lesser degree than in the case of RET. This is because in the absence of Complex I inhibitors, ROS production supported by NAD-linked substrates is also stimulated by high membrane potential. However, the dependence of ROS production rate on the amplitude of membrane potential is not so steep as in the case of RET.\textsuperscript{47}

It should be understood that there is no evidence directly supporting the hypothesis that mitochondrial Complex I (or in fact any other mitochondrial ROS producing site) is producing ROS \textit{in vivo}. All the evidence on ROS production by Complex I was obtained \textit{in vitro} with isolated mitochondria and \textit{extrapolated} to various \textit{in vivo} situations.
Complex I of the mitochondrial electron transport chain has been viewed as a major site of mitochondrial ROS production.\textsuperscript{32,39,50,51} There are three principal types of experiments that contributed to this concept: (a) experiments demonstrating that isolated Complex I preparations or submitochondrial particles generate ROS in the presence of NADH, (b) experiments with rotenone--inhibited mitochondria oxidizing NAD-dependent substrates, and (c) experiments with isolated mitochondria under conditions favoring RET from succinate to Complex I. The latter reaction generates large amounts of ROS.\textsuperscript{1,31} However, the possibility of RET under physiological conditions is not yet established. The interpretation of (b) and (c) -type experiments with intact mitochondria suffers from inherent uncertainty because the source of ROS could actually be something that is in a redox equilibrium with intramitochondrial NAD(P)H. This difficulty also applies to experiments demonstrating the dependence of mitochondrial ROS production on the amplitude of the membrane potential\textsuperscript{44–47} or intramitochondrial NAD(P)H/NAD(P)\textsuperscript{+} ratio.\textsuperscript{33,47} Logistically, such experiments do not allow one to quantify the contribution of Complex I to mitochondrial ROS production. The same argument applies to (a) -type experiments involving submitochondrial particles by their virtue of being mitochondrial fragments devoid of most of normal mitochondrial content and lacking normal mitochondrial enzyme interactions.

Therefore, whether or not Complex I is a significant source of ROS in intact mitochondria \textit{in vivo} is a complicated issue. There is even evidence that argues against the concept that Complex I in mitochondria, or in submitochondrial particles, can generate ROS at all, even in the presence of its inhibitors. The absence of a correlation between the inhibition of Complex I activity by rotenone and other inhibitors and the production of ROS by submitochondrial particles was interpreted as an indication of the presence of a superoxide--producing rotenone-binding site other than Complex I.\textsuperscript{52} The finding that H\textsubscript{2}O\textsubscript{2} production is frequently reported as being almost absent in the presence of succinate and rotenone,\textsuperscript{34,50} is intriguing because intramitochondrial NAD(P)H/NAD(P)\textsuperscript{+} ratio under such conditions is high. It is puzzling that Complex I does not generate ROS with at least the same efficiency under these circumstances, as observed with NAD-linked substrates with rotenone.\textsuperscript{50} Stimulatory effects of ADP\textsuperscript{50} and Ca\textsuperscript{2+} \textsuperscript{53–57} on mitochondrial ROS production are also intriguing because both Ca\textsuperscript{2+} uptake/retention, and ADP-induced oxidative phosphorylation dissipate energy and would
be expected to decrease the level of reduction of Complex I and hence, the ROS production. Even more intriguing is the fact that the stimulatory effect of the Complex I inhibitor rotenone on ROS production is in fact, species and tissue–dependent, as ROS stimulation by rotenone varies from \( \sim 300\% \) in guinea pig to 0\% in horse heart submitochondrial particles\(^{39}\) and in whole intact rat heart mitochondria,\(^{58}\) to inhibition of ROS production in mouse kidney mitochondria.\(^{21}\) Therefore, whether or not Complex I is a major site of ROS production continues to be a complicated issue indeed.

4. **Q-Cycle and the Mechanism of ROS Production at Complex III**

Historically, the first mitochondrial site producing ROS was identified at the Complex III (aka bc1 complex, ubiquinone:cytochrome c reductase) of the mitochondrial respiratory chain.\(^{59}\) The primary ROS produced at this site is superoxide,\(^{49,60–63}\) which quickly dismutates forming \( \text{H}_2\text{O}_2 \).\(^{64}\)

4.1. **The Q-cycle model of the coenzyme Q oxidation**

The scheme on Fig. 2A illustrates the mechanism of Complex III–catalyzed coenzyme Q (CoQ) oxidation known as the “Q-cycle.” The reaction starts from the oxidation of the CoQ quinol (\( \text{QH}_2 \)) in a bifurcated electron transfer reaction at the \( \text{Q}_o \)-site of the complex. The first electron is transferred to a high reduction potential chain consisting of the iron sulfur protein (ISP, or Rieske protein), cytochrome c1 (Cyt.c1) and cytochrome c (Cyt.c) and cytochrome c oxidase (not shown). This reaction leaves a semiquinone (\( \text{Q}^*_{o} \)), which is very unstable. This semiquinone donates the second electron to the low reduction potential chain consisting of two cytochromes b, \( \text{cyt} \ b_1 \) and \( \text{cyt} \ b_h \), which serve as a pathway routing the electrons to the \( \text{Q}_i \)-site. There, these electrons reduce another CoQ molecule. To provide two electrons required for the complete reduction of CoQ quinone at the \( \text{Q}_i \)-site, the \( \text{Q}_o \)-site oxidizes two \( \text{QH}_2 \) molecules in two successive turnovers. The first electron at the \( \text{Q}_i \)-site generates a stable semiquinone (\( \text{Q}^*_{i} \)) that is reduced to a quinol by the second electron.\(^{65–67}\)

Recently, the structures of bc1 complexes isolated from bovine, chicken, and rabbit mitochondria were determined by x-ray crystallography,\(^{66,68,69}\)
Fig. 2. The Q-cycle model of the coenzyme Q oxidation.

and the structural changes induced by the major inhibitors of the bc$_1$ complex were also determined. The data support the Q-cycle model. A novel finding of great importance is that the extramembrane domain of the ISP is mobile and undergoes a large scale movement to shuttle the electron from the quinol at the Q$_o$-site to the cytochrome c$_1$.\textsuperscript{66,68,69}

The scheme A indicates the sites of action of most frequently used inhibitors of bc$_1$ complex. The scheme B illustrates the mechanism of bc$_1$–catalyzed coenzyme Q (CoQ) oxidation known as the “Q-cycle”. The reaction starts from the oxidation of the CoQ quinol (QH$_2$) in a bifurcated electron transfer reaction at the Q$_o$-site of the complex. The first electron is transferred to a high reduction potential chain consisting of the iron sulfur protein (ISP, aka Rieske protein), cytochrome c$_1$ (Cyt.c$_1$) and cytochrome c (Cyt.c) and further to cytochrome c oxidase (not shown). The
remaining semiquinone ($Q_{o}^{•−}$) is unstable. It donates the second electron to the low reduction potential chain consisting of two cytochromes b, cyt $b_{l}$ and cyt $b_{h}$, which serve as a pathway conducting electrons to the $Q_{i}$-site. There, these electrons reduce another CoQ molecule. To provide two electrons required for the complete reduction of CoQ quinone at the $Q_{i}$-site, the $Q_{o}$-site oxidizes two $QH_{2}$ molecules in two successive turnovers. The first electron at the $Q_{i}$-site generates a stable semiquinone ($Q_{i}^{•−}$) that is reduced to a quinol by the second electron.$^{65–67}$

4.2. The site and source of electrons for the superoxide formation

An unstable semiquinone formed in the $Q_{o}$ center is believed to be the one-electron donor responsible for the superoxide formation.$^{35, 62, 63, 70–72}$ The semiquinone proposed to be present in the $Q_{o}$ center has, however, never been detected.$^{1, 31, 73}$ The effects of specific Complex III inhibitors played therefore the most important role for identification of both the site and the source of superoxide production.

Figure 2A shows the sites of action of three most frequently used inhibitors of Complex III. Myxothiazol prevents the binding of $QH_{2}$ at the $Q_{o}$-site, stigmatellin prevents the transfer of first electron to ISP, and antimycin A interrupts the transfer of the second electron to the $Q_{i}$-site.

The hypothesis that semiquinone in $Q_{o}$ center is the donor of electrons for the reduction of oxygen to superoxide is based primarily on the following experimental observations:

(1) The quinone of inner mitochondrial membrane is obligatory required for the antimycin A-induced superoxide production in bc1 complex.$^{62, 70}$

(2) The specific inhibitors of the bc1 complex affect the production of superoxide in a remarkable agreement with their effect on the formation of the putative semiquinone at the center $Q_{o}$. According to the classical Q-cycle hypothesis, inhibitors acting at the quinone-reducing center ($Q_{i}$), e.g. antimycin A, should stimulate superoxide formation by inhibiting semiquinone oxidation, as illustrated by the Fig. 2B. The inhibitor prevents the transfer of the second electron to the $Q_{i}$-site, thus “switching off” the low potential chain. This results in the accumulation of unstable semiquinone at $Q_{o}$-site and increases the probability of its side reaction with oxygen. However, the inhibitors of the $Q_{o}$ site, such as
myxothiazol or stigmatellin, should inhibit superoxide production by preventing semiquinone formation.\textsuperscript{1,31,65,67} Myxothiazol inhibits semiquinone formation at center Q\textsubscript{o} by displacing quinol at its binding site, whereas stigmatellin specifically blocks the first electron transfer reaction from quinol to ISP\textsuperscript{1,31,65,67} thereby preventing the semiquinone formation.

Therefore, antimycin A should stimulate the superoxide production as it was demonstrated,\textsuperscript{1,31} whereas myxothiazol should both prevent and inhibit the effect of antimycin. Indeed, myxothiazol was reported to inhibit superoxide production in mammalian mitochondria.\textsuperscript{44,62,71,72,74,75} Stigmatellin was also shown to both prevent and suppress the antimycin A–induced ROS production.\textsuperscript{76,77}

(3) Another observation strongly supporting both the Q-cycle hypothesis and that of superoxide production by the semiquinone in the center Q\textsubscript{o} was made by Turrens et al. These authors demonstrated that succinate-supported antimycin-induced H\textsubscript{2}O\textsubscript{2} production by the mitochondrial particles can be strongly inhibited by removing of cytochrome c from the particles, and restored by replenishing the cytochrome c. This observation can best be explained within the framework of the Q-cycle hypothesis, according to which the removal of the cytochrome c should prevent the oxidation of c1 and ISP and therefore the transfer of the first electron from the QH\textsubscript{2} (Fig. 2A) and thereby the formation of semiquinone at the center Q\textsubscript{o}. At the same time, this observation rules out both the ISP and the quinol as sources of ROS because they remain fully reduced in the absence of cytochrome c.\textsuperscript{72} Another observation made in the same study was that myxothiazol suppressed ROS production. It effectively excluded cytochromes b as reducers donating electrons for superoxide formation because both cyt. b\textsubscript{l} and cyt.b\textsubscript{h} remained fully reduced. Authors concluded that “by exclusion of other possibilities” ubisemiquinone at the center Q\textsubscript{o} was the only reduced electron carrier in complex III capable of producing superoxide.\textsuperscript{72}

(4) More evidence that superoxide is most likely produced by the oxidation of semiquinone at the center Q\textsubscript{o} was obtained in the studies of Dr. Konstantinov and colleagues. Using an EPR superoxide probe Tiron (1,2-dihydroxybenzo-3,5-disulfonate) they directly demonstrated superoxide production by inside-out submitochondrial particles reduced by succinate.\textsuperscript{63} These particles produced superoxide when inhibited by antimycin A (or a similarly acting inhibitor) but not when inhibited with
cyanide alone or with antimycin + cyanide, exactly as it would be expected if the superoxide was produced by the semiquinone at the center Qo.63 Further studies by Konstantinov’s group demonstrated that the effects of center Qo inhibitors mucidin, 2,3-dimercaptopropanol, and myxothiazol on the superoxide production were also exactly as expected, that is inhibitory.71,78

4.3. The unexplained features of the superoxide production mechanism at the Complex III

Earlier studies uncovered several puzzling features characterizing antimycin-inducible superoxide production at bc1-complex. While being relevant to the molecular mechanism of the superoxide production, all of them have yet to receive an explicit explanation.

Redox–dependence. The superoxide production\(^{a}\) by antimycin–inhibited submitochondrial particles exerts a bell-shaped dependence on the redox poise of the respiratory chain,71,77 rather than a sigmoidal dependence that would be expected for an unstable Qo-site allocated semiquinone.71 Such a redox behavior characterizes a stable semiquinone formed at equilibrium via a reversible dismutation of a quinone and a quinol, that is incompatible with an unstable semiquinone species at center Qo as a source of superoxide.71 The mechanism of this phenomenon was reported71 has been, and continues to be, under investigation in 1983.

Another peculiar recent observation is that myxothiazol\(^{b}\) can also induce ROS production by Complex III, albeit with a different redox-dependence and much lower rate of production than that in the presence of antimycin A.77 This observation was confirmed and expanded in experiments with isolated yeast bc1 complex79,80 and with isolated bovine and yeast bc1-complex.81 Muller et al. proposed a reasonable explanation for a shape of the redox-dependence of myxothiazol-induced ROS production and hypothesized that another semiquinone at Qo-center can be a source of electrons for the myxothiazol-induced superoxide formation.80

\(^{a}\) Measured as H\(_2\)O\(_2\) production.

\(^{b}\) This does not contradict a statement that myxothiazol suppresses antimycin-stimulated ROS production. Myxothiazol per se induces ROS production whereas it both prevents and inhibits the ROS production induced by antimycin.
“Wrong” sidedness of superoxide production. Earlier studies demonstrated that antimycin-induced superoxide production can be detected with submitochondrial particles (SMPs) but not with intact mitochondria.\textsuperscript{63,76} Given the complexity and high capacity of various mitochondrial ROS-destroying systems, failure of detecting superoxide production by the intact mitochondria is not overly surprising. It may be explained by the presence of SOD\textsuperscript{76,82} in the mitochondrial matrix; experimental artifacts such as a direct reaction of a superoxide probe Tyron with cytochrome c\textsuperscript{63,83} might also mask superoxide production. It is the release of superoxide by the inside-out SMPs that has to be somehow explained. The problem is that superoxide-generating Q\textsubscript{o}-site is located closer to the inner surface of the SMPs whereas a superoxide-detecting probe (a spin-trapping chemical or cytochrome c) is always outside of the particles. This disposition implies that either the probe or the superoxide molecules should be membrane-permeable. However, the study\textsuperscript{63} employed a negatively charged spin-trapping chemical Tyron which is unlikely to penetrate the SMP membrane;\textsuperscript{63} the negatively charged superoxide ion is also not expected to penetrate the membrane easily.\textsuperscript{c} Several studies demonstrated that mitochondria do release detectable Complex III-generated superoxide into the external space.\textsuperscript{82,89,90} However, these studies provide no explanation how the superoxide produced at the Q\textsubscript{o}-site can be released toward the matrix side of the mitochondrial membrane; neither have they allowed dismissing earlier observations with SMPs as erroneous.

The effect of uncouplers. Protonophorous uncouplers of oxidative phosphorylation such as FCCP stimulate the antimycin-induced H\textsubscript{2}O\textsubscript{2} production by mitochondria.\textsuperscript{70,91,92} Other energy-dissipating agents including Ca\textsuperscript{2+} and ionophores valinomycin and gramicidin also stimulate the H\textsubscript{2}O\textsubscript{2} production by isolated mitochondria.\textsuperscript{92} It was suggested\textsuperscript{70,92} that uncouplers stimulate the H\textsubscript{2}O\textsubscript{2} production by dissipating the small electrical potential across the mitochondrial membrane that still can be generated even

\textsuperscript{c}The ability of superoxide molecule to penetrate lipid membranes is circumstantial. Some studies demonstrated that superoxide can easily penetrate the plasma membrane of erythrocytes or even liposomes by means of an anion channel,\textsuperscript{84–86} whereas other studies found that the penetration of superoxide though the membranes of thylakoids and phospholipid liposomes is too slow or otherwise insignificant to be of any importance.\textsuperscript{86–88} To the best of our knowledge, the permeability of mitochondrial membranes to superoxide was not reported.
in the presence of antimycin. However, the mechanism of the membrane potential effect on the superoxide production by a semiquinone at Qo-center is not apparent. Under the experimental conditions of Cadenas and Boveris, uncoupling of mitochondria could stimulate the antimycin-induced H2O2 production by multiple mechanisms, e.g. by affecting the intramitochondrial succinate to fumarate ratio thereby shifting the redox poise of the respiratory chain (discussed above), or by increasing the permeability of mitochondrial membrane to protons thereby promoting the semiquinone reaction with oxygen. What makes the stimulatory effect of uncouplers interesting is that it apparently rules out a semiquinone at the center Qo of bc1 complex as the major site of ROS production by metabolically competent mitochondria. It is firmly established that high membrane potential stimulates whereas uncouplers strongly inhibit ROS production by coupled functional mitochondria (discussed elsewhere in this manuscript).

The pH dependence of ROS production. The maximum of the pH dependence of the superoxide and hydrogen peroxide production by antimycin-inhibited mitochondria or sonicated mitochondrial fragments is distinctly shifted toward alkaline conditions (pH > 7.5). Whereas this fact has never received an explanation, it might be of interest in regards to the role of this mechanism of ROS production under some pathological conditions. Obviously, any metabolic conditions acidifying a tissue milieu, such as lactic acidosis, would suppress this mechanism of ROS production by mitochondria.

The quinone concentration dependence of ROS production. ROS generation by antimycin-inhibited mitochondrial particles was shown to depend linearly on the amount of enzyme-reducible ubiquinone in the mitochondrial membrane. These experiments were performed with mitochondrial membranes that were extracted with acetone to remove most of endogenous ubiquinone. The extraction rendered the membrane particles practically incapable of both the electron transport from succinate to cytochrome c and the ROS production. Re-incorporation of various amounts of ubiquinone restored both activities. Surprisingly, the activity of succinate dehydrogenase (employed to reduce the re-incorporated quinone) and the rate of electron transport through the bc1 complex (measured as succinate-cytochrome c reductase activity, in the absence of antimycin)
were saturated at much lower amounts of re-incorporated ubiquinone than ROS production. The latter increased linearly with an increase in amount of succinate-reducible ubiquinone. It is also of interest that re-incorporation of less lipophylic ubiquinone-3 resulted in generation significantly less ROS than re-incorporation of more lipophylic ubiquinone-10.

4.4. The mechanism of superoxide production at Complex III

Despite the recent advances in understanding of the structure of the bc1 complex, a mechanism of superoxide production is not yet known. There is little doubt that semiquinone at center Qo is the most likely species responsible for the reduction of oxygen to superoxide (or even the only capable one); there are however, uncertainties about how it does it. This is primarily due to the fact that unstable semiquinone at the center Qo has yet to be demonstrated.

The published data allow for multiple models of Qo-site quinone occupancy, which significantly complicates the interpretation of the experimental data on the superoxide production at the site. Not a least important fact is that relatively little research efforts were invested in solving the mechanism of superoxide production per se; most data was obtained in attempts to prove the validity of Q-cycle scheme of electron transfer in Complex III.

Any molecular mechanism explaining how the superoxide is produced by the Qo-site originated semiquinone would have to account both for the known structural features of bc1 complex and for the unusual characteristics of the process as described above. An interesting recent idea is that the superoxide may be produced by a semiquinone that escaped from the Qo-site. However, we do not think that such an escape is possible in the absence of some severe conformational distortions of the bc1-complex resulting from binding of antimycin-like inhibitor or perhaps, a mutation affecting the Qo-site.

5. Mitochondrial ROS Detoxifying Systems

Earlier in this chapter, we described a struggle one little Holland boy endured against a hole in a dike that he sealed with his finger thus preventing
a flood. We further compared mitochondrial ROS production with that flood and described nine “holes”, the sources of ROS, that little mitochondrial Peter would have had to seal to save the day. Developing this analogy further, components of mitochondrial ROS-defense systems may be compared with Peter’s fingers. This section introduces ∼13 of them.

Decades–long fascination of researchers with the phenomenology of mitochondrial ROS production has shadowed the fact that mammalian mitochondria possess a complicated multi-leveled ROS defense network of enzymes and non-enzymatic antioxidants. The complexity of this network has just begun to be appreciated, and several new elements have been discovered recently. A systematic study of mitochondrial ROS defenses is yet to be performed, and a tissue-specific expression of many ROS-detoxifying enzymes is an additional complication to the understanding of its functioning. The enormity of the subject precludes us from providing a comprehensive review of mitochondrial ROS defenses. This chapter describes selected, primarily enzymatic subsystems (Fig. 3) that most likely represent mainstream mitochondrial ROS detoxifying pathways. It should however be kept in mind that the latter are yet to be established and that not all enzymes are present in mitochondria from every tissue.

5.1. Membrane lipid peroxide removal systems

The “perimeter” layer of ROS defenses is formed by the systems protecting lipids of mitochondrial membranes from peroxidation. These are chiefly α-tocopherol (TH) and phospholipid hydroperoxide glutathione peroxidase (where present). The TH is a ubiquitous lipid soluble directly operating non-enzymatic antioxidant dissolved in mitochondrial membranes. It can reduce lipid radicals “on contact” and requires regeneration for continuous operation. It can be regenerated by reduced coenzyme Q within mitochondrial membranes or by water–soluble ascorbic acid at the water/membrane interface. A physiological role, redox chemistry, tissue-specific distribution in mitochondria and other aspects of TH have repeatedly and comprehensively been reviewed elsewhere and will not be addressed here (see Packer96 for a recent review and Lass97 for distribution and content in rodents mitochondria).
5.2. *Phospholipids hydroperoxide glutathione peroxidase*

Phospholipids hydroperoxide glutathione peroxidase (PHGPx, aka GPx4, EC 1.11.1.12) is a mitochondrial selenoenzyme that belongs to the glutathione peroxidase family and utilizes glutathione. It catalyzes the reduction of phospholipid hydroperoxides to corresponding alcohols (Diagram 1), but it can also react with H₂O₂, cholesterol peroxides,⁹⁸,⁹⁹ and even with thymine peroxide.¹⁰⁰

![Diagram 1. Lipoperoxide Reduction Catalyzed by GPx4](image)

6. Lipoperoxide Reduction Catalyzed by GPx4

It is the only enzyme known to reduce peroxidized phospholipids within membranes and it is thought to play an important role in cellular ROS defense system.¹⁰¹ Homozygous knockout mice completely lacking GPx4 die *ab utero*, but heterozygous mice are viable and fertile.¹⁰² Mouse embryonic fibroblasts derived from GPx4 heterozygous animals were highly sensitive to paraquat, H₂O₂, tert-butylhydroperoxide, and gamma-irradiation.¹⁰² Overexpression of mitochondrial GPx4 in cells increased their resistance to several mitochondrial toxins inducing oxidative stress¹⁰³ and suppressed apoptotic changes including cytochrome c release from mitochondria, tentatively by inhibiting the peroxidation of a mitochondrial lipid cardiolipin.¹⁰⁴

GPx4 is synthesized in two isoforms, a short form and a long form (L-form) containing a leader sequence that is required for transport to mitochondria.¹⁰³ Detailed information on tissue distribution of mitochondrial (L-form) GPx4 is not available, except that it is absent in
mouse liver,\textsuperscript{105} and that L-form RNA transcript is present only in testis among murine tissues.\textsuperscript{106} In rat tissues, it is also highest in testis but some traces of L-form RNA transcript could also be detected in kidney, intestine, and cortex.\textsuperscript{107} Such narrow tissue specificity raises some doubts whether GPx4 is of any importance in mitochondria from tissues other than testis. In brain and testis mitochondria, GPx4 activity was localized in the inner mitochondrial membrane.\textsuperscript{108,109} It is however possible that the activity was due to other enzymes or a contamination with non-mitochondrial GPx4.

7. Superoxide Removal Systems

7.1. MnSOD

The second layer of ROS defenses is formed by enzymes dealing with primary ROS generated in mitochondria, superoxide radical and $\text{H}_2\text{O}_2$. The former is a substrate for mitochondrial manganese-containing superoxide dismutase (MnSOD, a.k.a. SOD2, EC 1.15.1.1). This enzyme is located exclusively inside the mitochondrial matrix; its only known function is to facilitate a dismutation of superoxide radical to $\text{H}_2\text{O}_2$ (Diagram 2), thereby protecting mitochondrial iron-sulfur cluster containing enzymes from the superoxide attack.\textsuperscript{110}

\[
\begin{align*}
2\text{O}_2^- + 2\text{H}^+ & \xrightarrow{\text{MnSOD}} \text{O}_2 + \text{H}_2\text{O}_2 \\
\text{Diagram 2.}
\end{align*}
\]

7.2. Superoxide removal by MnSOD

This fascinating enzyme is apparently very important because homozygous MnSOD knockout mice do not survive longer than a few days after birth.\textsuperscript{111,112} However, heterozygous mutant mice possessing only 50% of MnSOD activity and protein in their mitochondria are viable and fertile and do not develop any apparent abnormalities.\textsuperscript{111,112} A 50% deficiency in MnSOD did not result in an increased sensitivity to oxidative
Mitochondrial ROS Production

stress-promoting hyperoxia\textsuperscript{113} even when animals were exposed to lethal levels of oxygen.\textsuperscript{114} The MnSOD deficient mice live as long and age at the same rate as wild type mice despite having more accumulated DNA damage and cancer occurrence later in life.\textsuperscript{115} However, heart mitochondria isolated from these apparently healthy animals exerted signs of severe oxidative damage manifested as significant inhibition of mitochondrial Complex I and respiration with NAD-linked substrates, inhibition of aconitase, and increased sensitivity to Ca\textsuperscript{2+}-induced damage to mitochondrial integrity.\textsuperscript{116} Mitochondria isolated from hearts of MnSOD–deficient mice exerted \(\sim 2.4\%\) (\(\sim 4\) mV) higher membrane potential than mitochondria from wild type mice, that led authors to propose differences in the endogenous proton leak through inner mitochondrial membrane.\textsuperscript{116} Similar damage was found in liver mitochondria isolated from MnSOD deficient mice.\textsuperscript{117}

A sum of these and other data indicates that MnSOD is an important part of mitochondrial ROS defense system. It does not require any co-factors so the efficiency of this system in superoxide removal is determined by the amount of MnSOD enzyme present in mitochondria. The MnSOD activity is unevenly distributed among different tissues; in mice, the activity in liver and kidneys is highest followed by brain and heart, muscle, and spleen, with lungs exerting the lowest MnSOD activity, almost 20 times lower than that in liver.\textsuperscript{118}

Whereas heterozygous MnSOD deficient mice are apparently healthy, an overexpression of MnSOD to 6–10 times above the normal level resulted in developmental abnormalities and decreased fertility of mice.\textsuperscript{119} It is not clear what caused these abnormalities.

7.3. Cytochrome c

In addition to MnSOD, mitochondria possess another system capable of efficient superoxide removal. The intermembrane space of mitochondria contains \(\sim 0.7\) mM cytochrome c\textsuperscript{120} that can be alternatively reduced by either the respiratory chain or superoxide.\textsuperscript{121} This ability of cytochrome c to react with superoxide is well known and widely used to measure the superoxide production. The reduced cytochrome c is regenerated (oxidized) by its natural electron acceptor, cytochrome c oxidase (Diagram 3).
8. Superoxide Removal by Cytochrome c

The antioxidant properties of cytochrome c were demonstrated \textit{in vitro} in experiments with isolated mitochondria,\textsuperscript{122} but the physiological role and \textit{in vivo} efficiency of this superoxide-scavenging system remains to be explored. It deserves to be examined in detail also because if operational \textit{in vivo}, it would be the only known ROS-defense system that generates useful metabolic energy while detoxifying superoxide without producing toxic products. All other ROS–defense systems (except catalase) either consume energy for their regeneration or produce toxic products, such as H$_2$O$_2$. In contrast, oxidation of cytochrome c by cytochrome c oxidase generates protonmotive force that mitochondria can use to produce ATP, as was demonstrated in experiments with heart mitochondria exposed to the exogenously generated superoxide.\textsuperscript{123}

9. Hydrogen Peroxide Removal Systems

9.1. Catalase

The product of MnSOD reaction is H$_2$O$_2$ which \textit{per se} can be quite toxic to cells and mitochondria and has to be detoxified by other enzymes. One such enzyme is catalase (EC 1.11.1.6.), which converts H$_2$O$_2$ into O$_2$ and H$_2$O (Diagram 4).

\begin{center}
\begin{align*}
2\text{H}_2\text{O}_2 & \stackrel{\text{Catalase}}{\rightarrow} \text{O}_2 + \text{H}_2\text{O} \\
\end{align*}
\end{center}

Diagram 4.
In murine tissues, catalase activity is highest in liver followed by kidneys, lungs, heart and brain.\textsuperscript{124} It is thought that catalase is present only in heart mitochondria, where it comprises up to 0.025\% of all protein.\textsuperscript{125} The presence of catalase was also demonstrated in rat brain cortex mitochondria, where its content is developmentally regulated.\textsuperscript{126}

The role of catalase in mitochondrial ROS-defense network is not well understood. Even in heart mitochondria, the contribution of catalase to H$_2$O$_2$ removal is thought to be insignificant compared to that of glutathione peroxidase, another H$_2$O$_2$–detoxifying enzyme.\textsuperscript{127} The role of catalase in ROS-defenses in brain mitochondria is not known.

Recently, knockout mice lacking catalase activity were generated.\textsuperscript{124} These mice develop normally and do not show any apparent pathology. However, their brain mitochondria appeared to suffer more damage than mitochondria isolated from brains of wild type mice subjected to a physical impact brain injury. These experiments suggest that catalase may be dispensable under normal circumstances. In pathology, the role of catalase in ROS defense may be dependent on the type of tissue and the model of oxidant-mediated tissue injury.\textsuperscript{124}

\section*{9.2. Glutathione}

A staple of mitochondrial H$_2$O$_2$ defense network is a small tripeptide compound called glutathione. Glutathione (GSH, L-g-glutamyl-L-cysteinylglycine) is composed of cysteine, glutamic acid and glycine; its active group is the thiol (–SH) of cysteine. Various aspects of GSH metabolism, biochemistry, functions, and analysis have recently been extensively reviewed.\textsuperscript{128,129} Mitochondria contain \~{}10–12\% of total GSH amount in a cell, but due to their relatively small matrix volume the concentration of GSH in mitochondrial matrix is somewhat higher than that in cytosol.\textsuperscript{130} Mitochondria lack enzymes needed for GSH biosynthesis; intramitochondrial pool of GSH is replenished by rapid net uptake of GSH from cytosol.\textsuperscript{131--133} There are several systems capable of transporting GSH into mitochondria, including specialized low and high affinity GSH-transporters\textsuperscript{132} and dicaboxylate and 2-oxoglutarate carriers.\textsuperscript{133} On “average”, the concentration of glutathione within mitochondria is in the range from 2 to 14 mM\textsuperscript{130,131,134}; about \~{}90\% of glutathione is in its reduced
form, GSH.\textsuperscript{130,134,135} Actual concentrations of total (reduced + oxidized) glutathione in mitochondria vary depending on the metabolic state, age, and tissue.\textsuperscript{134} However, since published estimates for steady-state levels of H\textsubscript{2}O\textsubscript{2} in the matrix of mitochondria are in the low micromolar range,\textsuperscript{136} it is likely that even a significant decrease in GSH levels may not have an impact on H\textsubscript{2}O\textsubscript{2} detoxification by GSH–dependent enzymes. The question is then to what threshold level GSH can be depleted without impairing mitochondrial H\textsubscript{2}O\textsubscript{2} scavenging capacity. For rat heart mitochondria, the threshold level of GSH depletion was determined experimentally to be \(\sim 50\%\).\textsuperscript{137} An increase in mitochondrial H\textsubscript{2}O\textsubscript{2} emission was observed only after \(\sim 50\%\) depletion of GSH. After that threshold was reached, GSH loss corresponded to a linear increase in H\textsubscript{2}O\textsubscript{2} production by mitochondria.\textsuperscript{137}

9.3. Glutathione-S-transferase

Mitochondria utilize GSH in two major ways, as a recyclable electron donor and as a consumable in conjugation reactions.\textsuperscript{128} The latter are catalyzed by glutathione-S-transferases (GST, EC 2.5.1.18), several isoforms of which are present in mitochondria.\textsuperscript{138} These enzymes protect mitochondria from various toxins including products of lipid peroxidation such as 4-hydroxynonenal by adding a GSH molecule to a toxin; GSH is consumed and has to be replenished by the uptake from cytosol.\textsuperscript{131–133} A sufficiently large intramitochondrial pool of GSH ensures an efficient operation of a GST-based detoxifying system.

9.4. Glutathione reductase

Reduced glutathione can either scavenge superoxide and hydroxyl radical non-enzymatical or by serving as an electron-donating substrate to several enzymes involved in ROS-detoxifying.\textsuperscript{128} In either case, GSH is oxidized to GSSG that cannot be exported to cytosol\textsuperscript{139} and has to be reduced back to GSH in the mitochondrial matrix. The reduction is catalyzed by a specific enzyme glutathione reductase (GR, \textit{aka} GSSG reductase, \textit{aka} GSR, EC1.8.1.7, formerly EC1.6.4.2) which is present in the matrix of mitochondria.\textsuperscript{108,140–142} This enzyme utilizes intramitochondrial NADPH as a source of electrons for the reduction of GSSG to GSH (Diagram 5).
10. Glutathione Reduction by GR

In turn, mitochondrial NADPH can be regenerated by two major pathways, which are the substrate-dependent reduction by dehydrogenases of mitochondrial matrix and protonmotive force-dependent hydride ion transfer reaction utilizing intramitochondrial NADH to reduce NADP⁺. The former pathway is catalyzed primarily by NADP⁺-dependent isocitrate dehydrogenase (mNADP-IDH, aka IDPm, aka ICD1, EC 1.1.1.42) and by malic enzyme (NADP-ME, EC 1.1.1.40)¹³⁵; the latter is catalyzed by a protein of inner mitochondrial membrane, nicotinamide nucleotide transhydrogenase (TH, E.C.1.6.1.2).¹⁴³

10.1. A quintessence of the GSH-dependent mitochondrial ROS-defense network

The dual nature of NADPH regeneration pathways (Fig. 3) is a quintessence of the GSH-dependent mitochondrial ROS-defense network. It establishes the link between the mitochondrial ability to defend themselves against both endogenously and exogenously generated ROS, their bioenergetics prowess and oxidative capacity. In mitochondria, ROS detoxifying dissipates energy derived from a flow of carbon either directly, by oxidizing malate and isocitrate, or indirectly, by consuming protonmotive force generated by oxidation of any substrate (including malate and isocitrate). In either case, energy is spent to detoxify ROS instead of being used for other functions such as ATP synthesis. In either case, energy is used to regenerate NADPH
that is used to regenerate GSH that serves as an electron donor for various ROS-detoxifying systems. However, the enzymes involved in NADPH reduction are differentially expressed in various tissues thereby defining which pathway of GSH-regeneration in mitochondria would dominate in a specific mammalian tissue. It is conceivable that tissue specificity of GSH-regenerating pathways results in tissue-specific mitochondrial resistance to ROS or ROS-related toxin challenges.

Without knowing which pathway contributes more to NADPH reduction, it might be impossible to predict how mitochondrial ROS defenses would be affected by a toxin in different tissues. For example, a protonophorous uncoupler like 2,4-dinitrophenol (DNP) dissipates the protonmotive force thereby rendering TH–catalyzed NADP+ reduction inoperable. It may be anticipated that DNP would have more impact on mitochondrial GSH reduction level and ROS-defenses in mouse heart mitochondria than in brain mitochondria that express only 14% of TH than that in heart mitochondria.144 However, mouse brain mitochondria possess 3 to 7 times

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**Fig. 3.** Hierarchy of ROS-detoxifying systems in relation to the source of energy — Abbreviations: TCA, tricarboxylic acids cycle; RC, mitochondrial respiratory chain; GR, glutathione reductase; TrxR, thioredoxin reductase; GSH, reduced glutathione; Trx2 thioredoxin-2; GST, glutathione-S-transferase; GPx1 glutathione peroxidase-1; GPx4, phospholipid hydroperoxide glutathione peroxidase; Grx2 glutaredoxin-2; Prx3,5 peroxiredoxins 3 and 5.
higher activity of malic enzyme (depending on mouse strain)\textsuperscript{145} whereas their NADP isocitrate dehydrogenase activity is about 20 times lower than that of heart mitochondria.\textsuperscript{146} Both the accumulation of malate and isocitrate in mitochondria and their oxidation rate are individually controlled by the protonmotive force that is affected by the Uncoupler. Hence, the effect of DNP on mitochondrial NADPH, GSH and ROS defenses may be quite different depending on the comparative efficiency of all three NADP\textsuperscript{+} reduction pathways under specific experimental conditions for a specific tissue. Unfortunately, in real life such extended information is rarely available. Therefore, an effect of a toxin on mitochondrial ROS defenses has to be evaluated experimentally rather than assumed. On the bright side, the multiplicity of NADP reduction pathways ensures the robustness, flexibility and efficiency of mitochondrial GSH-linked ROS defense network.

\subsection{10.2. Hypothetical antioxidant function of NAD(P)H}

It should be noted that some authors hypothesize that NAD(P)H \textit{per se} can serve as a directly operating non-enzymatic antioxidant.\textsuperscript{147} Their reasoning is that mammalian mitochondria contain high concentrations of NADH and NADPH (\textasciitilde 3–5 mM of each),\textsuperscript{148} and that both NADH and NADPH readily react with oxygen-centered radicals such as trioxocarbonate and nitrogen dioxide, thereby scavenging them and preventing them from causing damage to mitochondrial proteins and DNA. Although such reactions usually result in formation of superoxide radical and further H\textsubscript{2}O\textsubscript{2}, and NAD(P)\textsuperscript{+} radical can further propagate ROS formation reactions, authors hypothesize that mitochondrial MnSOD and glutathione peroxidase are sufficient to prevent ROS build up.\textsuperscript{147} However, this original hypothesis should yet somehow account for a well-established fact that mitochondrial ROS production is strongly stimulated at high levels of NAD(P)H reduction.

\subsection{10.3. Glutathione peroxidase}

Classical glutathione peroxidase (GPx1, \textit{aka} cGPx, EC 1.11.1.9.) is likely the best studied mitochondrial enzyme that utilizes GSH for the reduction of H\textsubscript{2}O\textsubscript{2} to H\textsubscript{2}O (Scheme 5). This selenoenzyme is ubiquitously expressed in mammalian tissues\textsuperscript{149} and can be detected in various cellular compartments and in mitochondrial matrix\textsuperscript{108,140,150,151} and intermembrane space,\textsuperscript{108} the
same gene encodes both the mitochondrial and extramitochondrial GPx1. The enzyme is not specific toward its substrate and can react with both the H$_2$O$_2$ and organic hydroperoxides such as cumene hydroperoxide and tert-butyl hydroperoxide. The latter two compounds are frequently used to detect the enzyme activity in tissue samples in vitro. The glutathione peroxidase activity is high in liver, kidney and heart mitochondria and somewhat lower in brain and skeletal muscle mitochondria; however the detailed information on the expression and activity of GPx1 in mitochondria from different mammalian tissues is not available.

Some authors suggest that glutathione peroxidase is the most important enzyme in H$_2$O$_2$ removal even in heart mitochondria where catalase is present. Overexpression of Grx1 protected cells against various oxidants. Because of that, GPx1 was long thought to be an important part of cellular and mitochondrial ROS-defense network and a potential pharmacological target. These beliefs were shaken when it was discovered that homozygous knockout mice possessing no GPx1 activity are healthy, fertile, develop normally and do not show any signs of tissue damage and oxidative stress. That would imply that GPx1 is dispensable. However, other studies revealed that GPx1 knockout mice are significantly more sensitive than wild type mice to a number of toxins known to induce severe oxidative stress, including paraquat, N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, and 3-nitropropionic acid. Another study with knockout mice found mild growth retardation, slightly elevated H$_2$O$_2$ production and uncoupling in liver but not in heart mitochondria, and a remarkable absence of accumulation of lipid peroxides or any other signs of oxidative damage in heart and liver mitochondria from homozygous GPx1 knockout mice. The sum of data obtained with GPx1 knockout mice favor the idea that it is more involved in protection of tissues and mitochondria against acute oxidative stress induced by xenobiotics rather than being a major defense against low-level endogenous mitochondrial ROS production.

10.4. Peroxiredoxins and other oxins

Peroxiredoxins, or thioredoxin-dependent peroxide reductases, are recently discovered peroxidases that reduce H$_2$O$_2$ and lipid hydroperoxides (Diagram 6).
Two isoforms of peroxiredoxins (Prx or Prdx) were found in mammalian mitochondria, Prx3 and Prx5. Prx3 (aka SP-22) is ubiquitously present in mitochondria from various rat tissue, with the highest amount found in heart and adrenal tissue, followed by liver and brain.\textsuperscript{165} Similar Prx3 gene expression was found in bovine tissues except that it was highest in adrenal gland.\textsuperscript{166} Prx3 gene expression can be induced by oxidative stress; Prx3 apparently functions as an antioxidant in heart mitochondria\textsuperscript{167} and in neuronal mitochondria\textsuperscript{168} protecting them \textit{in vivo} against oxidative damage. However, the capacity and efficiency of Prx3 in H$_2$O$_2$ removal compared to those of other mitochondrial systems are not yet known.

![Diagram 6.](image)

Prx5 is the newest member of peroxiredoxins family discovered in mitochondria. Prx5 gene is also ubiquitously expressed in bovine tissues, with the highest level found in testis.\textsuperscript{166} Overexpression of human Prx5 in mitochondria of hamster ovary cells protected them from H$_2$O$_2$-induced oxidative damage thereby suggesting a role for this protein in mitochondrial ROS defense network.\textsuperscript{169}

A regeneration of both Prx3 and Prx5 to their active form is performed by mitochondrial disulfide oxidoreductase thioredoxin (Trx2) that is a part of so-called mitochondrial thioredoxin system. The backbone of the latter is composed of Trx2 that is a substrate for thioredoxin reductase (TrxR2) that in turn utilizes intramitochondrial NADPH as a hydrogen donor for the Trx2 reduction (Diagram 7).
Thioredoxin (Trx2)

$$\text{NADPH} + \text{H}^+$$

$$\text{Trx}_{2\text{Ox}}$$

$$\text{Protein}_{\text{Red}}$$

$$\text{Sh} \text{ HS}$$

$$\text{NADP}$$

$$\text{Trx}_{2\text{red}}$$

$$\text{Sh} \text{ HS}$$

$$\text{Protein}_{\text{Ox}}$$

Thioredoxin Reductase (TrxR2)

Thioredoxin system

Diagram 7.

Therefore, efficient operation of Prx3 and Prx5 is dependent upon an efficient regeneration of mitochondrial NADPH, similar to the GSH-linked systems described above. Glutaredoxin (Grx2) is also a member of this family of proteins and it can catalyze Trx-disulfide oxidoreduction reactions (Diagram 8).

$$\text{NADPH} + \text{H}^+$$

$$\text{Grx}_{2\text{Ox}}$$

$$\text{Protein}_{\text{Red}}$$

$$\text{Sh} \text{ HS}$$

$$\text{NADP}^+$$

$$\text{Grx}_{2\text{red}}$$

$$\text{Sh} \text{ HS}$$

$$\text{Protein}_{\text{Ox}}$$

Glutaredoxin system

Diagram 8.
However, it is different in that it can reduce both protein disulfides in dithiol reactions and catalyze monothiol reductions of mixed disulfides with GSH. Thioredoxins reduce efficiently only protein disulfides.

Thioredoxin and thioredoxin reductase (TrxR, EC1.8.1.9., formerly EC1.6.4.5.) and glutaredoxin are ubiquitous proteins present in many if not all tissues and performing a multitude of functions aside of their role in cellular antioxidant defenses. A wealth of information on tissue distribution, genetics, functions, reaction mechanism, and other aspects of these proteins is available. However, not much is known about specific mitochondrial isoforms of these proteins, Trx2, TrxR2, and Grx2, and even less is known about their specific role in mitochondrial ROS defenses. In murine tissues, different levels of mRNA transcripts for Trx2, Grx2, and TrxR2 were detected in spleen, lung, liver, kidney, brain, heart, and testis. The levels of Trx2, TrxR2 and Grx2 mRNAs were different from each other and did not follow any apparent pattern of coordinated transcription. The mitochondrial thioredoxin system seems to be essential for mammalian development because disruption of Trx2 gene in the mouse resulted in massive apoptosis during early embryogenesis and embryonic lethality. However, overexpression of Trx2 or TrxR2, or both, does not necessarily improve cell survival or resistance to ROS-promoting factors, indicating that perhaps an unidentified variable controls the effect of these proteins.

Summarizing this section, we should note that the role of these toxins disulfide reductases in mitochondrial ROS defenses remains to be explored. A wealth of information that is available regarding the antioxidant, cell signaling, and other important functions performed by non-mitochondrial isoforms of these proteins leaves no doubt that they can play a significant role in mitochondrial ROS defenses; that does not mean they do play that role. Instrumental might and wonders of modern day genetic engineering have proven that at least some of these proteins are obligatory for cell survival. That does not yet explain how their primary antioxidant functions provide for that cell survival, if the said functions have anything to do at all with that. There are plenty of examples when proteins perform several functions, with those that we do not know about being much more important than the one we can measure. Therefore, there are plenty of mitochondrial ROS-defense systems but their functioning as a fully integrated system remains to be explored. More experimental work is needed to reveal the...
roles and capacities of individual mitochondrial ROS-detoxifying systems in protection of mitochondria and cells from ROS.

11. Mitochondrial ROS Production in Pathologies

A compelling body of evidence indicates that oxidative stress is intimately involved in pathways leading to cell death and tissue damage. The role of endogenous ROS in etiology of various diseases and protective strategies have been extensively reviewed elsewhere and will not be covered here. Instead, we shall focus on the mitochondrially produced ROS and two least recognized aspects of the problem. The first aspect is a surprising shortage of studies where an increase in mitochondrial (as opposed to “source is not known”) ROS production associated with a disease was actually demonstrated rather than hypothesized. The second aspect that has just begun emerging in recent studies is an apparent controversy between the high energy requirements for ROS production by most known mitochondrial sites and a generally poor state of mitochondrial energy production observed in a pathology-affected tissue. The questions then arise — what are the mitochondrial sites that boost their ROS production in pathologies, and how does it happen. Regarding the highly popular emphasis placed on the role of mitochondrially produced ROS in almost every human disease including the most common one, ageing, the absence of answers on these questions comes as a bit of a surprise. In this chapter we review the state of the art in this field in light of the knowledge of mechanistic aspects of mitochondrial ROS production as described in previous sections.

11.1. Types of mechanisms enhancing mitochondrial ROS production

A multitude of mitochondrial sites that produce ROS suggest that there are many potential routes that could result in an increased ROS production by mitochondria in a pathology-affected tissue. However, all these routes could be arbitrarily divided into three major groups, one consisting of mechanisms (“active-type”) contributing to an increase in ROS production per se by mitochondria, the other uniting all the mechanisms (“passive-type”) contributing to a decrease in mitochondrial ROS scavenging capabilities, and
the third representing a special case when the two occur simultaneously. Although the “observable” outcome would always be an increase in an apparent mitochondrial ROS production, there are essential nuances.

The active-type mechanisms consume energy for ROS production, thus diverting a flow of reducing equivalents produced by oxidation of substrates to energy dissipation. ROS production by these mechanisms does double damage to a cell, by using huge catabolic capacity of mitochondria to generate harmful ROS while simultaneously decreasing the availability of energy needed for repairing ROS-induced damage and for ROS detoxifying. Obviously, such mechanisms would result ultimately in cell death unless compensated by an increase in activity/content of catalytic energy-independent ROS-detoxifying elements, such as catalase, MnSOD, cytosolic SOD, and in content of lipid and water-soluble antioxidants. The active-type mechanisms may for example include:

(i) a toxic intervention: a toxin-induced inhibition of Complexes I or III in mitochondrial respiratory chain or other redox-active enzymes preventing them from reacting with their physiological electron acceptors so that these complexes and enzymes would become over-reduced and prone to react with oxygen;

(ii) a mutational damage: a mutation-induced damage to the same complexes or other redox-active enzymes such that part of normal flow of electrons in these enzymes become diverted to superoxide production;

(iii) a peroxidative attack: anything inducing a chain-propagating peroxidation of mitochondrial lipids, such as iron loading of mitochondria;

(iv) a metabolic deregulation: anything elevating mitochondrial membrane potential above the normal level for that metabolic state, such as an ATPase mutation;

(v) an assembly failure: an improper assembly of mitochondrial redox-active multicomponent enzymes such as PDHC or KGDHC or respiratory chain complexes that would result in overall diversion of a normal electron flow toward ROS formation. Of course, any combinations of these mechanisms are possible, too.

The passive-type mechanism could perhaps be less damaging or even not lethal at all, thereby being responsible for a sustained elevation in mitochondrial ROS production and chronic oxidative stress. The passive-type mechanisms could play a major role in slow-developing diseases such as
various neurodegenerative conditions. These mechanisms could be difficult to detect in experiments with isolated mitochondria where a mitochondrial ROS production is measured; they should however be detectable by examining mitochondrial ROS-scavenging capacity\textsuperscript{142} or by directly assessing the content and activity of mitochondrial antioxidant systems.

The third type of the mechanism that is fairly relevant to various pathologies is a combination of both an active-type and a passive-type ROS producing mechanism. It is best represented by an increase in mitochondrial ROS production caused by mitochondrial permeability transition. It will be discussed in more detail later in this chapter.

11.2. Complex I as a site of enhanced ROS production

Unless induced by a toxin or a mutation-inflicted damage to Complex I, a poor state of mitochondrial bioenergetics is generally incompatible with an enhanced ROS production from Complex I of mitochondrial respiratory chain. The ROS production from this site requires high membrane potential that is hardly expected if the bioenergetics of mitochondria is suppressed. This argument is also valid for the lipoamide dehydrogenase, malate dehydrogenase, and other putative mitochondrial ROS-producing dehydrogenases that draw upon matrix pyridine nucleotides as their electron acceptors. The ROS production from these sites requires high NADH to NAD\textsuperscript{+} ratio that is not to be expected if the bioenergetics is poor. These are maladies of an excess, so to speak.

One may argue that it is not correct to limit a mutation-inflicted damage just to Complex I \textit{per se}, as from the previous sections it follows that interruption or suppression of electron flow at any site between Complex I and oxygen should stimulate ROS production. It is correct, however in that case ROS would be produced by all sites upstream of the site of inhibition, not by Complex I alone. However, it is conceivable that an inherited or acquired mutation in Complex I could result in a diversion of a normal electron flow within the complex toward an increased production of ROS. When such a mutation is found, it will be very interesting to examine what particular subunit of Complex I is involved in this elevated ROS production.

A deficiency in Complex I could be associated with an enhanced intracellular ROS production\textsuperscript{177–180}; however it is unlikely that it was
of mitochondrial origin. It was also demonstrated that a deficiency in NADH:cytochrome c reductase activity (that may reflect Complex I deficiency or some damage to Complex I) somehow resulted in an elevated superoxide production by isolated mitochondrial fragments oxidizing NADH\textsuperscript{177} \textit{in vitro}. Although there was no obvious correlation between the rate of ROS production and a degree of deficiency in NADH:cytochrome c reductase,\textsuperscript{177,179} these and other data do not contradict the hypothesis that a damage or a deficiency in Complex I \textit{in vivo} might indeed result in an elevated mitochondrial ROS production. Future experiments with mitochondria harboring a mutation in Complex I associated with Leber’s hereditary optic neuropathy may provide additional data in support of this hypothesis.\textsuperscript{181}

11.3. Complex III as a site of enhanced ROS production

Enhanced ROS production by the mitochondrial Complex III would perhaps not require high membrane potential or a high NAD(P)H/NAD(P)+ ratio in the mitochondrial matrix. However, the mitochondrial Complex III-derived ROS production in the absence of antimycin or a similar inhibitor is yet to be demonstrated. In fact, the sum of available \textit{in vitro} data on the mechanism of ROS production at this site and the absence of confirmatory \textit{in vivo} data clearly indicate that while it could have produced ROS \textit{in vivo}, it obviously does not, unless severely damaged by antimycin A, myxotiazol, or other inhibitor. Nevertheless, it is conceivable that many man-made environmental, agricultural, and habitual toxins might be able to cause an antimycin-like inhibition of Complex III and stimulate mitochondrial ROS production in an affected tissue; these toxicities just await to be demonstrated.

A deficiency in Complex III activity originating from a mutation in mtDNA in a patient with Parkinsonism resulted in an increased intracellular ROS production in transmitochondrial cybrids.\textsuperscript{182} Future experiments should establish whether that increase was due to mitochondrially produced ROS or due to a decrease in mitochondrial ROS scavenging capacity resulting from impaired bioenergetics associated with diminished Complex III activity.\textsuperscript{182}
11.4. **ATPase mutation may enhance ROS production**

A mutation in this or that mitochondrial enzyme that enhances mitochondrial ROS production has yet to be demonstrated. However, a mitochondrial mutation that results in an elevated steady state level of cytosolic ROS has been recently demonstrated. A T8993G point mutation in mtDNA targets one of the subunit (MTATP6) of mitochondrial ATPase and impairs oxidative phosphorylation in two mitochondrial disorders.\(^{183}\) Experiments with transmitochondrial cybrid cells harboring this mutation revealed higher cytosolic ROS, impaired mitochondrial ATP synthesis, and elevated mitochondrial membrane potential.\(^{183}\) Although mitochondrial ROS production per se was not measured, it is not unlikely that a mutation inhibiting an energy-dissipating process (oxidative phosphorylation) would increase the membrane potential thereby stimulating mitochondrial ROS production.

11.5. **Ischemia reperfusion enhances mitochondrial ROS production**

There exists an acute shortage in relevant published experimental data regarding the mitochondrial ROS production in diseases and pathologies. Although a deficiency in this or that mitochondrial enzyme would frequently result in impaired bioenergetics, it does not necessarily mean an enhanced mitochondrial ROS production. Similarly, an increase in cytosolic steady-state ROS levels does not necessarily mean that it originates from mitochondria, even if it appears to respond on mitochondrial inhibitors. In almost all studies of oxidative stress associated with pathology the mitochondrial ROS production per se has not actually been examined.

A notable exception is an elevated mitochondrial ROS production associated with ischemia and reperfusion-induced tissue damage. Ischemia-reperfusion associated ROS production has been studied most extensively in heart tissue where it is manifested by three phenomena: an elevated ROS production during the ischemia phase, a “burst” in ROS production upon the onset of reperfusion that fades in a few minutes, and an elevated ROS production observed in reperfused tissue.\(^ {184}\) It is well established that mitochondria isolated from either ischemic or reperfused heart tissue exert an enhanced ROS production compared to mitochondria isolated from control tissue.\(^ {185–188}\) A significant decrease in Complex III
activity was also observed, that perhaps contributed to a conclusion that Complex III is responsible for the enhanced ROS production. However, it would be more likely that ROS was actually generated by Complex I and/or dehydrogenases in mitochondrial matrix. This is because mitochondrial ROS production was measured in the presence of Complex I inhibitor rotenone and succinate as oxidative substrate; these conditions are known to stimulate ROS production by Complex I and matrix dehydrogenases. Other studies report a significant decrease in NAD-linked respiration and/or Complex I activity and increased ROS production by mitochondria isolated from ischemic or reperfused heart tissue. The measurements were performed under conditions favoring increased ROS production by Complex I and/or matrix dehydrogenases (State 4 respiration supported by NAD-linked substrates).

It is not known what causes a decrease in Complex I activity and an increase in ROS production from this site in mitochondria during ischemia and reperfusion. An accumulation of long chain unsaturated fatty acids such as arachidonic acid may be the factor responsible for both phenomena. Another likely mechanism could be the opening of mitochondrial permeability transition pore during the reperfusion phase (reviewed later in this chapter), because most of the results obtained with ischemic mitochondria were also obtained with control mitochondria that were allowed to accumulate Ca$^{2+}$. Apparently, in situ tissue conditions and/or factors affecting mitochondria are not obligatory for stimulating mitochondrial ROS production by reperfusion. Isolated liver mitochondria subjected to anoxia in vitro exerted an elevated superoxide production upon reperfusion. Authors suggested that it was caused by the oxidation of ubisemiquinone that could have accumulated during the anoxic phase; it is however clear that something else could be equally responsible for such a burst as everything redox-capable in mitochondria becomes over-reduced during the anoxia phase.

It should be noted that ischemia-induced increase in mitochondrial ROS production may be a tissue-specific phenomenon. A study failed to detect an increase in ROS production by mitochondria isolated from rat brain subjected to a post-decapitative ischemia; no decline in mitochondrial Complex I or III activities was noted either. However, their experimental model was different from those where an increase in mitochondrial
ROS production was observed in that the ischemia was not followed by a reperfusion.\textsuperscript{190}

**11.6. Mitochondrial Ca\(^{2+}\) accumulation per se unlikely enhances ROS production**

There are numerous reports implying that a massive mitochondrial accumulation of Ca\(^{2+}\), another prominent phenomenon associated with ischemia and reperfusion,\textsuperscript{191,192} somehow promotes ROS production.\textsuperscript{53,54,57,193,194}

The Ca\(^{2+}\) uptake per se should suppress ROS production because it dissipates the $\Delta \Psi$ and decreases the level of NAD(P)H reduction in mitochondria. It also induces collateral energy expenditures caused by Ca\(^{2+}\) recycling and re-phosphorylating of ATP hydrolyzed during the phase of active transport. This reasoning was proved experimentally; ROS production by mitochondria oxidizing NAD-linked substrates was severely suppressed both during the active Ca\(^{2+}\) uptake and for a prolonged period after the accumulation has been completed.\textsuperscript{195}

It is also unlikely that Ca\(^{2+}\) accumulation could stimulate ROS production by affecting dehydrogenases in mitochondrial matrix. Although Ca\(^{2+}\) in the low micromolar range stimulates the activity of several dehydrogenases including pyruvate dehydrogenase complex, isocitrate dehydrogenase, and $\alpha$-ketoglutarate dehydrogenase complex,\textsuperscript{196,197} a massive Ca\(^{2+}\) accumulation actually inhibits the activity of the very same dehydrogenases\textsuperscript{198,199} as well as the overall NAD-linked respiratory activity and phosphorylation in mitochondria.\textsuperscript{200,201} Upon reperfusion, Ca\(^{2+}\) is accumulated in mitochondria well above normal, physiological matrix concentration.\textsuperscript{191,192} Therefore, it is unlikely that Ca\(^{2+}\) effect on matrix dehydrogenases is responsible for Ca\(^{2+}\)-associated stimulation of ROS production observed upon reperfusion of ischemic tissues.

**11.7. Ca\(^{2+}\)-induced mitochondrial permeability transition may be responsible for an increase in ROS production**

The most dramatic pathological event associated with over-accumulation of Ca\(^{2+}\) by mitochondria is the opening of a large pore in the inner mitochondrial membrane. This is a unique mitochondrial phenomenon that was extensively studied for over 20 years yet is far from being well-understood.
Mitochondrial permeability transition pore (PTP) is thought to be a large channel in the inner mitochondrial membrane which is normally closed and can be opened by Ca\(^{2+}\) overloading and other factors including oxidative stress. Structural changes such as partial release of the cytochrome c typically accompanies the PTP\(^{202-206}\) as well as loss of mitochondrial matrix pyridine nucleotides\(^{207}\) and other solutes such as glutathione.\(^{208}\) The various characteristics of PTP and its importance and involvement in etiology of various diseases and in cell death are extensively reviewed elsewhere.\(^{202,205}\)

Several reports demonstrate that opening of PTP correlates with an increase in ROS production by isolated mitochondria\(^{56,209}\) and in cells.\(^{210}\) It is conceivable that PTP-induced changes in mitochondrial structure and content are the major reason for increased ROS production observed in ischemia-reperfusion models. It is also most likely the main mechanism for an increase in mitochondrial ROS production in other pathologies that are associated with abnormal Ca\(^{2+}\) regulation, such as glutamate neurotoxicity.\(^{210}\) Another prominent example of a condition where PTP opening is responsible for an increase in mitochondrial ROS production is so-called “ROS-induced ROS release”. This is an interesting phenomenon described by Dr. Zorov et al.\(^{211}\) that consist of an increase in mitochondrial ROS production induced by an exposure of mitochondria to increasing levels of exogenously generated ROS. In the experiments of Dr. Zorov and colleagues, ROS were generated incrementally and spatially in the mitochondria-rich regions \textit{in situ} in intact myoblasts by a laser irradiation of a photoactive ROS-producing chemical. Several mitochondrial parameters including their membrane potential, ROS production, and PTP opening were monitored simultaneously using a confocal microscopy technique. It appeared that photodynamically ROS triggered the PTP opening in mitochondria that resulted in depolarization of the membrane potential and secondary increase in mitochondrial ROS production.\(^{211}\) This study is of primary importance as it is the first direct demonstration of \textit{in situ} ROS production by mitochondria that had undergone PTP opening.

According to the classification presented earlier in this chapter, PTP-induced ROS production represents a combination of both the active and the passive-type mechanisms. A PTP opening in mitochondrial inner membrane should induce a genuine increase in ROS production from several mitochondrial sites, primarily from Complex I and substrate dehydrogenases of mitochondrial matrix. This is illustrated in Fig. 4. Small
Fig. 4. Effect of massive Ca\(^{2+}\) accumulation and mitochondrial permeability transition on ROS production. See text for details.
solute with the molecular weight less than 1,500 Da are released from mitochondrial matrix upon the onset of PTP. This includes matrix pyridine nucleotides,\textsuperscript{207} that are released downward the gradient of their concentration, as mitochondrial concentrations of NAD and NADP are $\sim$10 times higher than their concentrations in a cell cytosol.\textsuperscript{148} However, the substrates of mitochondrial dehydrogenases are still available, and this results in over-reduction of substrate dehydrogenases in the mitochondrial matrix due to the lack of their natural electron acceptor (pyridine nucleotides). This would stimulate ROS production by the NAD-linked enzymes such as dihydrolipoamide dehydrogenase and malate dehydrogenase, as described in a previous section of this manuscript. Complex I is also expected to increase its ROS production because a PTP opening induces partial loss of cytochrome c from mitochondria thereby inhibiting the respiratory chain and inducing Complex I over-reduction.

All the active mitochondrial antioxidant systems become dysfunctional shortly after the PTP occurs. This is because in the absence of proton gradient and sufficient supply of NADH and NADPH mitochondrial reduced glutathione cannot be regenerated and is eventually depleted, in addition to its direct release into cell cytosol downward the concentration gradient.\textsuperscript{130} This results in an impaired ROS scavenging by mitochondria.

\section*{12. Postscriptum}

A huge wave in interest in mitochondrial free radicals production mechanisms is growing internationally. Hopefully, it will deliver advanced knowledge about the functioning and regulation of mitochondrial ROS generating and detoxifying systems and their role in the life and death of cells. Research on mitochondrial ROS production and detoxifying mechanisms has been a side-bar, rather than a mainstream subject of modern biology for far too long. The subject of mitochondrial free radicals has not yet fully embraced the power of post-genome era tools and conceptions and hypothesis-driven approaches. Nevertheless, even with the present patchwork state of knowledge, the emerging complexity and degree of interactions in mitochondrial ROS-related systems is impressive. With carefully designed and conclusive advances in our understanding of these complex and integrated systems, the
potential significance of mitochondrial ROS in regulating tissue bioenergetics and pathogenesis may deserve distinct recognition as critical control points in Maps of Biochemical Pathways.

References


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