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Review

Redox Control of Mitochondrial Functions

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ABSTRACT

Redox reactions and electron flow through the respiratory chain are the hallmarks of mitochondria. By supporting oxidative phosphorylation and metabolite transport, mitochondrial redox reactions are of central importance for cellular energy conversion. In the present review, we will discuss two other aspects of the mitochondrial redox state: (i) its control of mitochondrial Ca^{2+} homeostasis, and (ii) the intramitochondrial formation of reactive oxygen or nitrogen species that strongly influence electron flow of the respiratory chain. Antioxid. Redox Signal. 3, 515–523.

REDOX REGULATION IN MITOCHONDRIA: THE CASE OF Ca^{2+} HOMEOSTASIS

Reactive oxygen species formation and the redox barrier in mitochondria

REACTIVE OXYGEN SPECIES (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), the hydroxyl radical ($^{\cdot}OH$), and singlet oxygen are produced during normal metabolism (13, 16). In the early 1970s, Chance and co-workers (16) reported that H_2O_2 is produced by the respiratory chain. The discovery of superoxide dismutase (SOD) in mitochondria (99) prompted the search for O_2^- in mitochondria. Loschen et al. (59) were the first to show mitochondrial O_2^- production and to document that the constitutively formed mitochondrial H_2O_2 originates from O_2^-.

Mitochondria consume >90% of the oxygen used in the body. Although most of the oxygen is fully reduced to water at the level of cytochrome oxidase (COX), a fraction of it is incompletely reduced to O_2^- at the level of other respiratory sites (Fig. 1). Early reports suggested that 1–2% of the total oxygen is converted into O_2^- (16); however, there is no general consensus in this regard (23).

The steady-state concentrations of mitochondrial O_2^- and H_2O_2, the predominant precursors of the highly reactive $^{\cdot}OH$, are estimated to be in the picomolar and nanomolar range, respectively. In general, the main O_2^- generators in mitochondria are the ubiquinone radical and NADH dehydrogenase (53, 98). However, according to Nohl (68, 69), cytochrome *b* rather than ubisemiquinone is a site of O_2^- production. O_2^- is formed by autoxidation of the reduced components of the respiratory chain. This explains the increased O_2^- production with increased oxygen pressure, rotenone, or antimycin, and the higher O_2^- production in state 4 compared with the state 3 of respiration.

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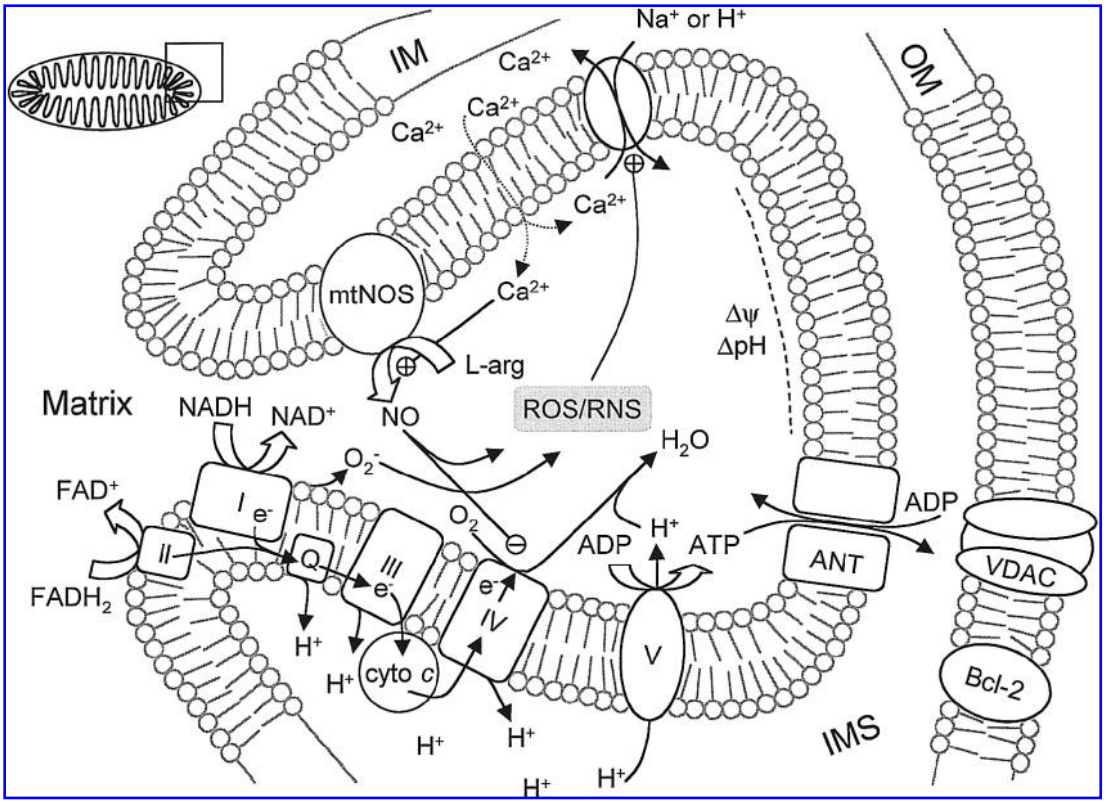

FIG. 1. Mitochondrial functions, mitochondrial nitric oxide synthase, and reactive oxygen/nitrogen species. Mitochondrial respiratory complexes are embedded in the mitochondrial inner membrane (IM). Electrons enter the chain from the complexes I or II, flow down to complex IV, and reduce oxygen to water. Coupled to the electron flow, respiratory chain complexes extrude protons into the mitochondrial intermembrane space (IMS), which produces a transmembrane potential ($\Delta\Psi$) and a ΔpH across the coupling membrane. The influx of protons through complex V, ATP synthase, is the driving power for ATP formation from ADP. The adenine nucleotide translocator (ANT) transports ATP in exchange for ADP from mitochondrial matrix into the IMS. Mitochondrial nitric oxide synthase (mtNOS) is located in mitochondrial IM and produces nitric oxide (NO) from L-arginine (L-arg) in a Ca^{2+}-dependent manner. Hereby, mitochondrial Ca^{2+} uptake follows the membrane potential. NO regulates mitochondrial respiration via reversible inhibition of complex IV, cytochrome oxidase. Intramitochondrially formed NO readily reacts with oxygen radicals such as O_2^- to produce other reactive oxygen/nitrogen species (ROS/RNS) such as $ONOO^-$, which stimulates mitochondrial Ca^{2+} release. Intramitochondrially formed ROS/RNS also induce apoptosis, via, *e.g.*, release of cytochrome *c* (cyto *c*) from the IMS into the cytosol, in a not fully known manner. Once released from the IMS, cyto *c* plays key roles in the cellular apoptotic pathway. Mitochondrial respiratory chain complexes are shown as boxes, I–V. OM, outer membrane; Q, ubiquinone Q 10; VDAC, voltage-dependent anion channel; ⊕, stimulation; ⊖, inhibition.

In addition to the normal ROS production in mitochondria, reactive oxygen is formed in large amounts in the presence of certain compounds, *e.g.*, so-called "redox cyclers" such as alloxan, and during some pathological states, *e.g.*, diabetic hyperglycemia (67).

Mitochondrial antioxidants

The harmful effects of ROS produced in mitochondria are normalized by several mitochondrial redox, antioxidant, barriers (101). Mitochondrial O_2^- and H_2O_2 are metabolized in mitochondria by the Mn-containing SOD (Mn-SOD) and the Se-containing glutathione peroxidase, respectively (16). Heart mitochondria, however, contain catalase (73). They produce considerable amounts of H_2O_2 per gram of tissue, because of the high mitochondrial content and almost exclusively aerobic metabolism of myocardial cells. Skeletal muscle mitochondria, which produce H_2O_2 at a rate equivalent to 40% of that of heart mitochondria, seemingly do not contain catalase (72). ROS are furthermore scavenged by the vitamin antioxidants (66, 96), glutathione (88), and ubiquinol-10 (22,

25). McCord and co-workers (39) proposed that the respiratory chain activity could establish a sink for O_2^-. According to their view, respiring mitochondria present a polarized proton-rich surface that promotes nonenzymatic dismutation of extramitochondrial O_2^-. Despite the efficient antioxidant defense systems, oxidative damage to mitochondria is abundant.

Mitochondria and cellular Ca^{2+} homeostasis

The intracellular concentration of free Ca^{2+} is four orders of magnitude less than its extracellular concentration. Alterations of the intracellular Ca^{2+} level regulate many processes. This highly regulated Ca^{2+} concentration is adjusted by its binding to nonmembranous mitochondrial proteins, and to membrane-bound Ca^{2+}-ATPases located primarily in the plasma, nuclear, and endoplasmic reticular membrane.

Mitochondria are of central importance for physiological Ca^{2+} handling: They are Ca^{2+} reservoirs, and provide much of the ATP used by Ca^{2+}-ATPases. Ca^{2+} also regulates the activity of intramitochondrial dehydrogenases, as well as nucleic acid and protein synthesis (75).

The importance of mitochondria as short-term modulators of cytosolic Ca^{2+} under physiological conditions was overlooked until recently. However, there is now compelling evidence (40, 82) that during physiological cell stimulation mitochondrial Ca^{2+} transport directly participates in the modulation and maintenance of cellular Ca^{2+} homeostasis. Several reports have additionally documented that physiological cytosolic Ca^{2+} pulses are relayed into mitochondria of brain, liver, and *Xenopus laevis* oocytes (44, 58, 89). More recently, experiments underlined the importance of mitochondrial Ca^{2+} transport, which modulates the amplitude and spatiotemporal organization of Ca^{2+} in the cytosol, in cell signaling. Particularly the Ca^{2+}-induced release of Ca^{2+} from mitochondria allows the organelles to generate and convey electrical and Ca^{2+} signals (42, 45). The pivotal role of mitochondria in store-operated Ca^{2+} current was recently discussed (35). These organelles are also able to take up large amounts of Ca^{2+} and, by buffering cytosolic Ca^{2+}, act against potentially toxic levels of Ca^{2+} in the cytosol.

Redox regulation of the mitochondrial Ca^{2+} release

Ca^{2+} can leave mitochondria by three routes: by a nonspecific leakage of the inner membrane, by reversal of the influx carrier, and by a Na^+-dependent or -independent release pathway (14, 20). The latter two are specific and redox-dependent and operate with preserved mitochondrial transmembrane potential ($\Delta\Psi$) (Fig. 1). The Na^+-dependent pathway predominates in mitochondria of heart, brain, skeletal muscle, adrenal cortex, brown fat, and most tumor tissue. The Na^+-independent pathway is important in liver, kidney, lung, and smooth muscle mitochondria, probably exchanges Ca^{2+} with H^+. These specific Ca^{2+} release pathways are regulated by the redox state of mitochondrial pyridine nucleotides.

Prooxidant-induced, NAD^+-linked mitochondrial Ca^{2+} release

The regulation of mitochondrial Ca^{2+} release by the redox state of mitochondrial pyridine nucleotides was first described by Lehninger's group (54), who showed that enzymatic oxidation of NAD(P)H by acetoacetate or oxaloacetate promotes mitochondrial Ca^{2+} release, whereas reduction of $NAD(P)^+$ by β-hydroxybutyrate in the presence of rotenone prevents it. Shortly thereafter, it was reported that hydroperoxides, such as *tert*-butyl hydroperoxide and H_2O_2, promote mitochondrial Ca^{2+} release by oxidation of pyridine nucleotides, followed by hydrolysis of NAD^+ to ADP-ribose and nicotinamide (60, 61). Since then, many prooxidants were identified that stimulate Ca^{2+} release from liver, heart, brain, and kidney mitochondria secondary to pyridine nucleotide oxidation and hydrolysis, followed by protein mono-ADP-ribosylation (78).

The redox state of mitochondrial pyridine nucleotides, the hydrolysis of NAD^+, and therefore ADP-ribosylation and Ca^{2+} release, are under the control of vicinal thiols: Phenylarsine oxide, which reversibly forms a five-membered ring with vicinal thiols, promotes the Ca^{2+}-dependent intramitochondrial NAD^+ hydrolysis and thereby the specific Ca^{2+} release (87). Gliotoxin, a fungal metabolite carrying a disulfide moiety, also promotes the Ca^{2+}-dependent

intramitochondrial NAD^+ hydrolysis and thereby the specific Ca^{2+} release, but is inactive when its sulfurs are reduced or methylated (84). Thus, intramitochondrial, Ca^{2+}-dependent NAD^+ hydrolysis is prevented when some vicinal thiols are reduced, in the SH form, and occurs when they are oxidized, connected, either by a cross-linking reagent or by oxidation to the disulfide form. NAD^+ hydrolysis and therefore Ca^{2+} release stimulated by *tert*-butyl hydroperoxide are prevented by 4-hydroxynonenal (HNE) with an initially unclear mechanism (77). It was later established (47) that HNE prevents also the gliotoxin-induced Ca^{2+} release in a manner sensitive to thiols and their pK_a. For example, preincubation of HNE with glutathione at high, but not at low, pH reduces inhibition of Ca^{2+} release by HNE. These findings give further evidence to that mitochondrial functions, such as Ca^{2+} homeostasis, are controlled by the redox state of mitochondrial thiols. Nitric oxide (NO) is produced in mitochondria (27). It reacts with SH moieties to produce S-NO proteins, and with O_2^- to form peroxynitrite ($ONOO^-$), which avidly oxidizes thiols and its congeners (see below).

REDOX REGULATION IN MITOCHONDRIA: THE CASE OF NO AND ITS CONGENERS

Biology of NO

The finding of the obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine (26), followed by the discovery of NO (43, 71), opened a new window in the field of biomedical research. It changed our opinion of NO from a toxic gas to that of an important beneficial molecule in biology (49). At physiologically relevant concentrations, NO binds to COX, the terminal enzyme of the mitochondrial respiratory chain (Fig. 1), and thereby deenergizes mitochondria in a transient, reversible, and cyclic GMP-independent manner (85). Besides its physiological actions, NO also participates in some pathophysiological events. The important roles of NO and $ONOO^-$, the product of its stoichiometric reaction with O_2^-, in ischemia/reperfusion injury, atherosclerosis, sexual dysfunction, and neurodegenerative diseases have been broadly investigated (5, 6, 21, 34, 52, 55, 56, 74, 91).

NO is synthesized by NO synthase (NOS; EC 1.14.13.39) isozymes. Although none of the members has a tissue-specific pattern of expression, the family of NOS is categorized in three extensively characterized isozymes, the constitutively expressed neuronal (nNOS) and endothelial (eNOS), and the inducible (iNOS) isoform (62). Constitutive NOSs are strongly Ca^{2+}-dependent and show a typical interaction with calmodulin, whereas the activity of iNOS does not increase when the cytosolic Ca^{2+} rises, although calmodulin is necessary for its activity. NOS isozymes are dimeric hemoproteins with a monomer molecular mass of ~126–160 kDa, which use L-arginine and molecular oxygen to produce NO and L-citrulline. Production of NO by NOS regulates many systems. For example, NO keeps blood pressure lowered and acts as the retrograde messenger in memory formation and as a cytotoxic molecule in immune defense (65).

NO regulates mitochondrial functions

A considerable part of the biological properties of NO is mediated via its interaction with mitochondria. The best demonstrated target of NO in mitochondria is COX, the terminal enzyme of the respiratory chain. The inhibition by NO of COX is demonstrated at various levels, including isolated enzyme, submitochondrial particles, mitochondria, hepatocytes, nerve terminals, and astrocytes (10, 11, 15, 18, 79, 85, 93, 94). NO binds to the oxygen binding site of the reduced COX and, therefore, competes with O_2 (12, 80, 97) in a manner that resembles a pharmacological competitive antagonism. Why this inhibition is transient is not clear at the moment, but it has been suggested that NO is consumed at the level of COX (17). COX can reduce NO (102), and NO can combine with O_2 to form NO_x, or with O_2^- to form $ONOO^-$. Very recently we suggested that $ONOO^-$ might be formed at the level of COX (32). Interestingly, concentrations of NO needed for inhibition of COX and mitochondrial respiration are close to what have been measured in

many biological systems (9). NOS inhibition results in the stimulation of respiration in many systems (7, 11, 41, 57, 92–94, 100). Besides COX, NO also affects other mitochondrial respiratory complexes. It has been demonstrated that NO inhibits complex I by *S*-nitrosylation of its critical thiols (19) in a manner reversed by glutathione, the mitochondrial major redox molecule.

Between 1995 and 1997, the possible existence of a mitochondrially located NOS was investigated by several groups. Immunohistochemical studies using NADPH diaphorase staining (3, 24) and silver-enhanced gold immunolabeling (4) provided evidence for the presence of NOS-like proteins in several locations within the mitochondria. Colocalization of eNOS with succinate dehydrogenase as a mitochondrial marker (48), and cross-reaction of mitochondria with eNOS (3, 4) or nNOS antibodies (24) were reported. One study (48) also reported faint L-arginine-dependent NOS activity in a preparation of rat diaphragm muscle mitochondria, but did not rule out the presence of nonmitochondrial NOS, nor the influence of the urea cycle in citrulline formation, which was used as the only indicator of NOS activity. In 1997, for the first time, the presence of a constitutively expressed and continuously active NOS in mitochondria [mitochondrial NOS (mtNOS)], its localization in the inner mitochondrial membrane (Fig. 1), and determination of its activity (27, 81) were reported. It was shown that mtNOS is Ca^{2+}-dependent and that the enzyme substantially controls mitochondrial respiration and $\Delta\Psi$. Soon thereafter, the presence of mtNOS and its association with the inner mitochondrial membrane was confirmed (2, 37), and the enzyme was purified (95).

Knowing that NO exerts a wide range of its biological properties via regulating mitochondrial functions, the finding that mitochondria carry their own NOS opened a new window to the NO research fields. mtNOS stimulation causes a decrease in mitochondrial oxygen consumption and $\Delta\Psi$ and, consequently, it was concluded that mtNOS stimulation should decrease ATP synthesis (27). This conclusion was later experimentally supported (36). Interestingly, incubation of mitochondria with conventional NOS inhibitors increases mitochondrial basal oxygen consumption and $\Delta\Psi$ (27–29, 33). It shows that with endogenous mitochondrial substrates and cofactors and at basal mitochondrial Ca^{2+} levels, mtNOS is continuously active and regulates mitochondrial respiration and, therefore, cellular energy production.

The mitochondrial respiratory complexes are embedded in the inner mitochondrial membrane, and electron flow in the respiratory chain is accompanied by extrusion of protons from the matrix into the mitochondrial intermembrane space, which creates a ΔpH and $\Delta\Psi$ across the inner membrane (Fig. 1). Recently, we reported that NO, provided by an NO donor or produced by mtNOS, acidifies mitochondrial matrix and that inhibition of mtNOS causes alkalinization of mitochondrial matrix and provides resistance to the sudden drop in $\Delta\Psi$ induced by mitochondrial Ca^{2+} uptake (28). The $\Delta\Psi$ is essential for many mitochondrial functions, such as protein and cation, *e.g.*, Ca^{2+}, import. NO, added exogenously (85) or produced by mtNOS (27, 33), inhibits COX and thereby decreases $\Delta\Psi$. Interestingly, a drop in $\Delta\Psi$ occurs when cells undergo apoptosis (64).

Mitochondria play a central role in apoptosis (29, 30, 76). Many apoptogenic factors, such as ceramide, induce apoptosis *via* interaction with mitochondria and release of mitochondrial cytochrome *c* (31). Identification of mtNOS and its location near the respiratory chain, where O_2^- is formed, make it highly likely that $ONOO^-$ is formed within mitochondria. On the other hand, an increase in the cytosolic Ca^{2+} level caused by, *e.g.*, glutamate or Fas receptor stimulation, is apoptogenic (63). Many recent reports show that mitochondrial Ca^{2+} uptake is an essential step in Ca^{2+}-induced apoptosis (1, 50, 90). This kind of programmed cell death is accompanied by increased NOS activity (1, 90) and is prevented by MnSOD (38, 46), or by the $ONOO^-$ scavenger, urate (51). Mitochondria produce NO in a Ca^{2+}-dependent manner and are rich O_2^- sources. Recently, we showed that upon mitochondrial Ca^{2+} uptake and mtNOS stimulation, mitochondrial cytochrome *c* is released in a Bcl-2-sensitive manner (33) and that mtNOS-induced cytochrome *c* release is due to intramitochondrial $ONOO^-$ formation. It is sug-

gested that prolonged elevation of Ca^{2+}-induced apoptosis is, at least partly, mediated via stimulation of mtNOS.

Given that mitochondrial Ca^{2+} uptake and release are regulated by $\Delta\Psi$, it seems very probable that mtNOS participates in mitochondrial and thereby cellular Ca^{2+} homeostasis. As mentioned above, mitochondria are essential components of phasic cellular Ca^{2+} responses, *e.g.*, Ca^{2+}-induced Ca^{2+} release (83). The exact molecular mechanism, however, is not fully elucidated. It is known that $ONOO^-$ stimulates mitochondrial Ca^{2+} release (70, 86) and that $ONOO^-$ is formed intramitochondrially (8, 33). Very recently, we showed that accumulation of mitochondrial Ca^{2+}, which leads to mtNOS stimulation and intramitochondrial $ONOO^-$ formation, causes the release of mitochondrial Ca^{2+} *via* a cyclosporin A-sensitive and pyridine nucleotide-dependent pathway, followed by mtNOS deactivation (8). Thus, mitochondrial Ca^{2+} release in response to increased cytosolic Ca^{2+} concentration may be a part of a physiological feedback loop preventing the overloading of mitochondria with Ca^{2+}. In contrast to NO, $ONOO^-$ does not reversibly inhibit mitochondrial respiration. Mitochondrial O_2^- and SOD, by modulating the steady-state concentrations of NO and $ONOO^-$, may therefore have a hitherto undetected impact on cellular Ca^{2+} homeostasis.

CONCLUSION

It is known since the pioneering work done in the 1950s and 1960s that redox reactions drive ATP synthesis and transport processes in mitochondria. More recently, the control of mitochondrial Ca^{2+} homeostasis by the redox state of pyridine nucleotides and thiols has become apparent. The discovery of NO formation in mitochondria, its dependence on Ca^{2+}, and the consequences for respiratory chain activity are other important aspects of redox control in mitochondria.

ABBREVIATIONS

$\Delta\Psi$, mitochondrial transmembrane potential; COX, cytochrome oxidase; eNOS, endothelial nitric oxide synthase; HNE, 4-hydroxynonenal; H_2O_2, hydrogen peroxide; iNOS, inducible nitric oxide synthase; MnSOD, manganese-containing superoxide dismutase; mtNOS, mitochondrial nitric oxide synthase; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; O_2^-, superoxide; $^{\cdot}OH$, hydroxyl radical; $ONOO^-$, peroxynitrite; ROS, reactive oxygen species; SOD, superoxide dismutase.

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