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Forum Review

Production of Reactive Oxygen Species in Brain Mitochondria: Contribution by Electron Transport Chain and Non–Electron Transport Chain Sources

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ABSTRACT

Overwhelming evidence has accumulated indicating that oxidative stress is a crucial factor in the pathogenesis of neurodegenerative diseases. The major site of production of superoxide, the primary reactive oxygen species (ROS), is considered to be the respiratory chain in the mitochondria, but the exact mechanism and the precise location of the physiologically relevant ROS generation within the respiratory chain have not been disclosed as yet. Studies performed with isolated mitochondria have located ROS generation on complex I and complex III, respectively, depending on the substrates or inhibitors used to fuel or inhibit respiration. A more "physiological" approach is to address ROS generation of *in situ* mitochondria, which are present in their normal cytosolic environment. Hydrogen peroxide formation in mitochondria *in situ* in isolated nerve terminals is enhanced when complex I, complex III, or complex IV is inhibited. However, to induce a significant increase in ROS production, complex III and complex IV have to be inhibited by >70%, which raises doubts as to the physiological importance of ROS generation by these complexes. In contrast, complex I inhibition to a small degree is sufficient to enhance ROS generation, indicating that inhibition of complex I by ~25–30% observed in postmortem samples of substantia nigra from patients suffering from Parkinson's disease could be important in inducing oxidative stress. Recently, it has been described that a key Krebs cycle enzyme, α-ketoglutarate dehydrogenase (α-KGDH), is also able to produce ROS. ROS formation by α-KGDH is regulated by the NADH/NAD^+ ratio, suggesting that this enzyme could substantially contribute to generation of oxidative stress due to inhibition of complex I. As α-KGDH is not only a generator but also a target of ROS, it is proposed that α-KGDH is a key factor in a vicious cycle by which oxidative stress is induced and promoted in nerve terminals. *Antioxid. Redox Signal.* 7, 1140–1149.

INTRODUCTION

There is overwhelming evidence that oxidative stress is involved in the pathogenesis of the progressive deterioration of neurons associated with aging, neurodegenerative diseases, excitotoxicity, or ischemia/reperfusion (3, 6, 36, 42, 58, 72, 74). The fact that mitochondria are the major sites for generation of reactive oxygen species (ROS) in cells (23) placed mitochondria in the center of interest, and much evidence has accumulated implying that mitochondrial defects play a crucial role in the pathogenesis of these conditions by initiating and promoting oxidative stress.

During normal cellular respiration oxygen is consumed by mitochondria, and at the terminal step of the oxidative phosphorylation machinery it is reduced to H_2O. Electrons travel along the respiratory chain, which is composed of four complexes: complex I (NADH ubiquinone oxidoreductase), complex II (succinate ubiquinone oxidoreductase), complex III (ubiquinone-cytochrome *c* oxidase), complex IV (cytochrome *c* oxidase), plus ubiquinone and cytochrome *c*. The ATP synthase is also part of the oxidative phosphorylation machinery and is called complex V. The complexes are arranged in the inner membrane of mitochondria in a strict order determined by the redox potential of the individual components. The res-

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piratory chain is "leaky," meaning that electrons could escape from this route and reduce O_2, resulting in the generation of superoxide ($O_2\cdot^-$), the primary ROS. The nonenzymatic production of superoxide occurs by a one-electron reduction of oxygen requiring reduced coenzymes or prosthetic groups. In the respiratory chain, there are a variety of redox centers, which are thermodynamically able to provide one electron for oxygen, including iron-sulfur clusters, flavoproteins, and ubisemiquinone. In addition to the respiratory chain, monoamine oxidase located in the outer membrane of mitochondria could also be a significant source of ROS production, in particular, in dopaminergic neurons, where the physiological turnover of dopamine by monoamine oxidase involves the generation of hydrogen peroxide (H_2O_2). Nonetheless, the major site of superoxide generation in the mitochondria is generally assumed to be the respiratory chain and, until recently, (93, 96) no other sources of ROS in the mitochondria have been seriously considered. In recent years, a number of reviews covered different aspects of oxidative stress (1, 6, 33, 82, 97, 100). This review focuses on the role of the respiratory chain and Krebs cycle in the ROS generation of brain mitochondria.

Superoxide produced in the mitochondria has a very short half-life as it is dismutated by manganese superoxide dismutase (Mn-SOD) in the mitochondrial matrix (37) or by copper/zinc superoxide dismutase (Cu,Zn-SOD) in the intermembrane space and the cytosol giving rise to the formation of H_2O_2 (38, 61). H_2O_2 is also a ROS, although not a radical, which is relatively membrane-permeable and able to leave the mitochondrial matrix and, when produced in excess, is released from the cell. In the presence of transition metals, hydroxyl radical (·OH) is generated from H_2O_2 and superoxide, which is the most reactive ROS. Normally, H_2O_2 is eliminated by glutathione or it is converted to H_2O in the enzyme reactions by catalase or glutathione peroxidase/glutathione reductase present in both the mitochondrial matrix and the cytosol (Fig. 1). The presence of catalase may be tissue-specific; it has not been found in mitochondria prepared from skeletal muscle (81). In brain mitochondria, the activities of glutathione peroxidase/glutathione reductase are relatively low (84), but they do play a significant role in the elimination of H_2O_2 in brain tissues (31, 32). Due to the antioxidant defense in the mitochondria, the steady-state concentration of superoxide and H_2O_2 is around 10^{-10} and 5×10^{-9} *M*, respectively (17).

Among ROS, H_2O_2 can be detected most reliably, and as it is membrane-permeable, H_2O_2 released from the mitochondria or cells can be measured as a stoichiometric indication of superoxide production (10, 62). Indeed, in most of the studies addressing mitochondrial ROS production or generation of ROS in cells, H_2O_2 is determined. For this, either chemiluminescent or fluorescent assays (mostly Amplex Red) are applied (71, 110). Alternatively, the activity of aconitase, which is inhibited by ROS, can be used as a sensitive marker of superoxide (39) or H_2O_2 (95) production.

It is believed that, even during normal oxidative phosphorylation, 1–4% of oxygen is reduced in mitochondria by a one-electron reduction to generate superoxide (9). The succinate-supported H_2O_2 release from brain mitochondria was estimated to be 3% of the total oxygen consumption in the study by Cino and Del Maestro (27). In a recent study, this value was calculated to be 1.5%, whereas in mitochondria supported by glutamate and malate 0.2% of the consumed oxygen was estimated to contribute to superoxide production (53).

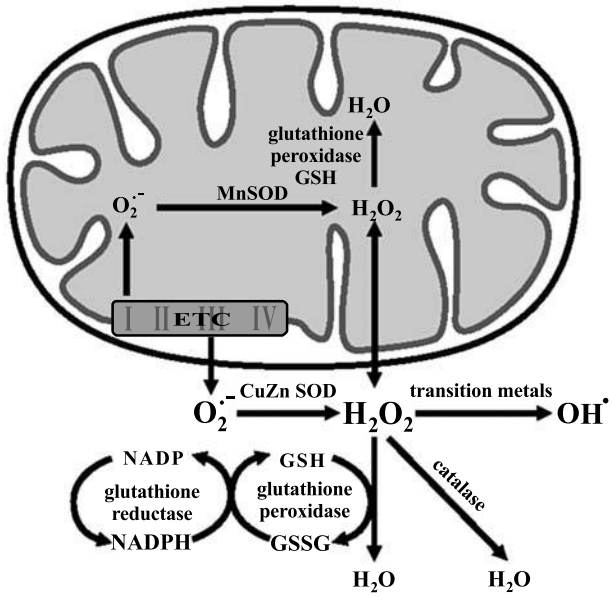

FIG. 1. Elimination of superoxide ($O_2\cdot^-$) and hydrogen peroxide (H_2O_2). Antioxidant enzymes are present in the mitochondria and the cytosol. ETC, electron transport chain; GSH, reduced glutathione; GSSG, oxidized glutathione; SOD, superoxide dismutase.

THE SITE OF ROS GENERATION IN THE RESPIRATORY CHAIN: STUDIES ON ISOLATED MITOCHONDRIA

The exact site and mechanism of a physiologically important ROS generation in the respiratory chain have not been established yet. There is a great deal of variability in the literature concerning the rate and substrate specificity of superoxide and H_2O_2 production in mitochondria, and results are sometimes controversial and difficult to compare. In the majority of studies, isolated mitochondria prepared from different tissues were used, which is a very convenient and attractive preparation for studying the mechanism of ROS generation, as with different substrates and inhibitors of the respiratory chain the site of superoxide production can be addressed. The variability of results could arise from a number of differences in the experimental protocols used for detecting superoxide or H_2O_2. In this respect, the importance of the O_2 saturation in the medium has been recently pointed out (53). The rate of generation of H_2O_2 is clearly dependent on oxygen concentration (2, 11, 27, 53, 102, 103); thus, due to a low oxygen saturation in the medium used in many studies, the calculation of ROS production could be inaccurate.

In addition, it is evident that there are major differences in the characteristics of ROS production in mitochondria derived from different tissues and species. It is particularly noteworthy that characteristics of ROS generation in neuronal

and nonneuronal mitochondria are in many respects different. Some of the differences will be pointed out in this review with an emphasis on ROS generation in neurons.

For identification of superoxide-producing sites in isolated mitochondria, different substrates fueling the respiratory chain via either complex I or complex II and/or inhibitors of the individual components of the respiratory chain have been used (Fig. 2). With glutamate and malate as substrates providing mainly NADH, the respiratory chain receives electrons at complex I, whereas with succinate providing $FADH_2$, electrons are given to complex II. It is to be noted that, due to the function of the Krebs cycle, some entry of electrons at complex II might also occur in the presence of NADH-linked substrates, and vice versa, complex I can be fueled to some extent when succinate is used as a substrate. With inhibitors present, the flow of electrons in the respiratory chain slows down and the upstream components become more reduced, favoring the escape of electrons and the formation of ROS. The most generally used inhibitors in these studies are rotenone blocking complex I and antimycin A blocking complex III and, in some studies, cyanide inhibiting complex IV. To have a deeper insight into the mechanism of superoxide generation, additional complex inhibitors, such as myxothiazol and stigmatellin, have also been used (see below).

In isolated heart and rat liver mitochondria, large amounts of H_2O_2 generation were detected in the presence of glutamate/malate (11, 12, 59, 60) or succinate (11, 12). Early studies performed mainly on mitochondria derived from nonneuronal tissues suggested two sites for superoxide production in the respiratory chain, namely, the Q cycle of complex III and an unknown site in complex I (13, 18, 101, 103). In heart mitochondria, the major site of ROS production was proposed to be complex III based on the observation that when oxidizing complex I substrates, heart mitochondria produced a minimal amount of H_2O_2 and rotenone failed to enhance ROS generation, whereas antimycin A markedly increased H_2O_2 generation with both complex I and complex II substrates (24).

Data from different studies are controversial as to the generation of ROS in isolated brain mitochondria under resting conditions (in the absence of inhibitors). Earlier, no evidence was found for ROS production here (91), but later numerous investigations revealed that mitochondria derived from the brain do generate ROS during resting conditions (27, 79, 111). It has become clear that the rate of ROS generation is different when complex I substrates are used compared with that found in the presence of the complex II substrate. With NADH-linked substrates, no H_2O_2 generation under resting conditions, in the absence of electron transport chain inhibitors, was reported in some studies (27, 59, 104), but in others a detectable amount of H_2O_2 was found (53, 79). However, inhibition of complex I by rotenone or that of complex III by antimycin provoked the generation of H_2O_2 (27, 45, 92, 104, 111).

Succinate, however, is a very effective ROS-generating substrate for isolated brain mitochondria (44, 54, 59, 104). Pioneer investigations concerning mitochondrial ROS generation suggested that the ubiquinone site of complex III is responsible for the ROS generation in mitochondria supported by succinate (18, 103). Later, however, it was clarified that the succinate-supported H_2O_2 generation in brain mitochondria was sensitive to inhibition by rotenone, indicating that the one-electron reduction of oxygen in succinate-supported mitochondria involves a reverse electron flow from succinate to the NADH-dehydrogenase (27, 59, 104). The phenomenon of reverse electron flow in mitochondria was first described by Chance and Hollunger (22), and the importance of this pathway in the ROS production by mitochondria supported by succinate was reinforced by the finding that inhibitors of the succinate-supported NADH formation (reverse electron flow), such as ADP, FCCP (carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone), and malonate, all blocked the succinate-induced ROS production (59).

Reverse electron flow requires a tightly coupled mitochondrial membrane and is very sensitive to changes in pH (56,

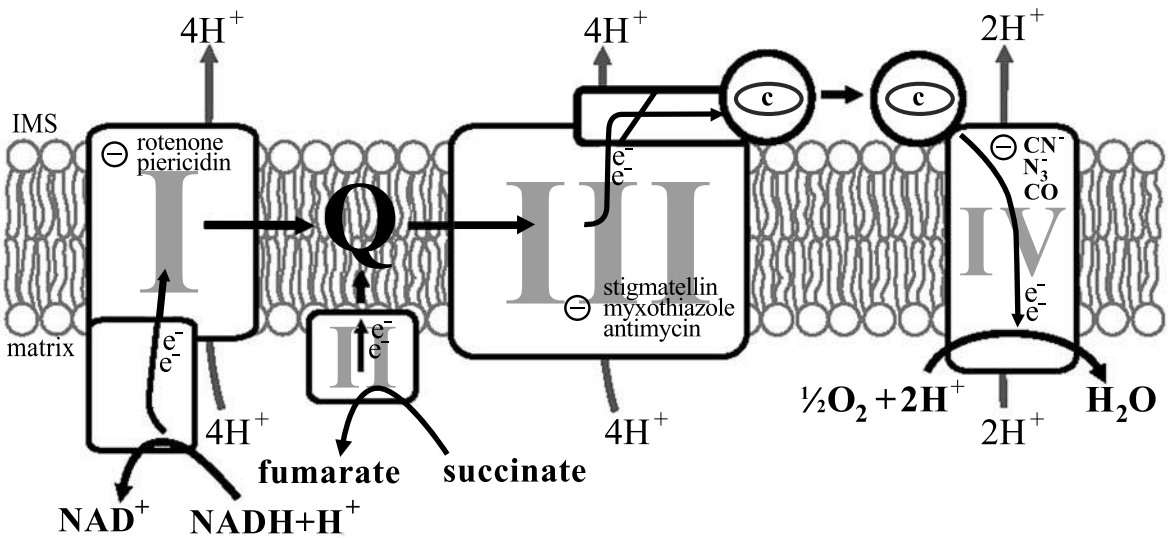

FIG. 2. Complexes of the mitochondrial respiratory chain. The flow of electrons is indicated by arrows. Inhibitors of the individual complexes are shown (⊖). IMS, intermembrane space.

57) and mitochondrial membrane potential ($\Delta\Psi_m$) (50). In agreement with this, succinate–induced ROS generation was inhibited by a very small degree of depolarization in isolated brain mitochondria (104). It is evident that the reverse electron flow is not physiological; therefore, the role of this process in the ROS generation *in vivo* appears to be unlikely.

The mechanism of superoxide production in succinate-supported mitochondria is different when antimycin is also present, revealing ROS production in the forward reaction. ROS generation in the presence of antimycin has been suggested to occur at the ubiquinone site of complex III (18, 103). In isolated rat brain mitochondria, the presence of an uncoupler prevented the succinate-supported H_2O_2 generation, but antimycin in the presence of the uncoupler was able to stimulate the release of H_2O_2 by mitochondria (27, 104), indicating that when complex III is inhibited by antimycin, the coupling state of mitochondria is not critical for the generation of superoxide.

THE MECHANISM OF ROS GENERATION AT COMPLEX I OR COMPLEX III

Complex I is the first component of the electron transport chain transferring electrons from NADH to ubiquinone. It is a large macromolecule composed of 46 subunits containing a series of iron-sulfur centers (19, 80, 105). Electrons from NADH first reduce the flavin-containing subunit flavin mononucleotide [(FMN)], then are transferred via a series of subunits containing iron-sulfur centers, and eventually reduce ubiquinon (Q) to ubiquinol (QH_2) involving the formation of semiquinone ($QH^{\cdot -}$). The exact mechanism and site of superoxide generation at complex I have not been fully clarified, and FMN (53, 59, 109), the iron-sulfur (40, 45, 99, 101), and semiquinone (45, 56, 82) have been assumed to be the electron donors for the superoxide formation (Fig. 3).

Rotenone blocks complex I at a distal site, near or at the binding site for ubiquinone (76). An increase in ROS generation by inhibition of complex I in mitochondria supported by NADH-linked substrates points to a site of electron leak at or proximal to the rotenone binding site. When rotenone inhibits complex I, the proximal sites of the complex become fully reduced, favoring the electron leak to oxygen. It has been suggested that mitochondria oxidizing NADH substrates generate ROS in the presence of the complex I inhibitor rotenone at the iron-sulfur centers (99, 101). However, the effect of diphenyleneiodonium on the H_2O_2 release from brain mitochondria indicated that the FMN group, rather than the iron-sulfur centers of complex I, is responsible for the ROS generation (59). This flavin-modifying compound selectively inhibits complex I (but not complex II) both *in vitro* and *in vivo* (46). When applied in succinate-supported mitochondria, it inhibited the production of H_2O_2, without affecting the activity of complex II; thus, it was suggested that the succinate-supported ROS production occurs at the FMN group, the first electron carrier of complex I (59). This was supported by the finding that the midpoint potential of superoxide production in submitochondrial particles equaled −295 mV, in agreement with the midpoint potential of FMN, but not with that of iron-sulfur clusters, and that the flavin-modifying agent, diphenyleneiodonium inhibited the rotenone-insensitive, oxygen-dependent NADH oxidation in brain mitochondria (53). Alternatively, it has been reported that a high rate of superoxide production by complex I in the forward reaction requires a pH gradient across the mitochondrial inner membrane and is induced only when Q-site inhibitors are present (rotenone, piericidin, or high concentrations of myxothiazole), promoting the reaction of, most likely, semiquinone with oxygen (56).

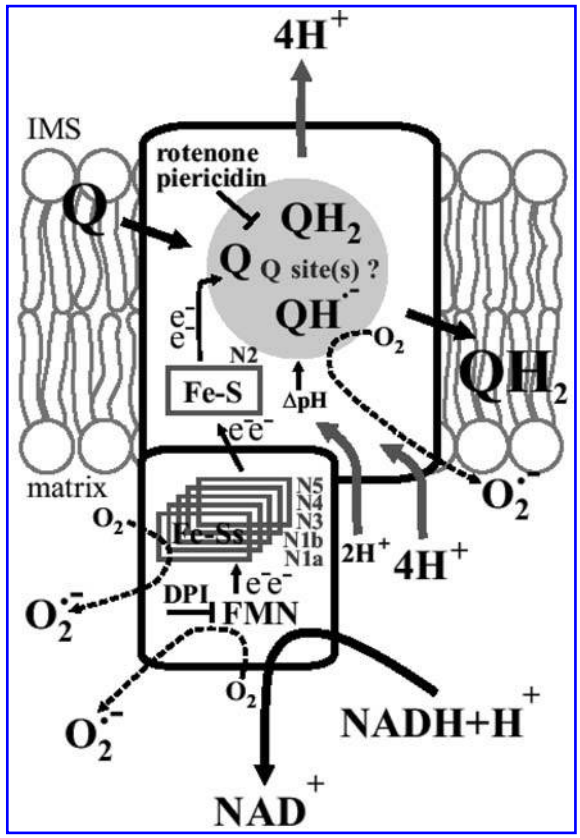

FIG. 3. Possible sites of superoxide ($O_2^{\cdot -}$) production on complex I. The possibility of superoxide generation by the flavin-containing subunit, the iron-sulfur centers, and semiquinone is indicated. DPI, diphenyleneiodonium; Fe-S, iron-sulfur centers; FMN, flavin-containing subunit; IMS, intermembrane space; Inhibitors acting on the Q site are indicated. Q, ubiquinone; $QH^{\cdot -}$, semiquinone; QH_2, ubiquinole.

It has been suggested that as the electron leak from complex I is located in the inner mitochondrial membrane on the matrix side, superoxide produced at this site is released into the matrix where it can be more readily eliminated by antioxidant enzymes, including Mn-SOD (24). This is consistent with the observation made earlier (59, 111) that in glutathione-depleted mitochondria supported by NADH-dependent substrates, the amount of H_2O_2 released in the presence of rotenone is significantly increased.

In heart and lung mitochondria, complex III appears to play the most important role in the generation of superoxide (101, 102). This is also the site of superoxide formation in

succinate-supported brain mitochondria in the presence of antimycin (59, 111), which is almost completely inhibited by myxothiazole (104, 111). Complex III is partially responsible for the ROS generation in glutamate/malate-supported mitochondria as suggested by some (104, 111), but not all studies (27). However, the physiological importance of ROS generation at complex III in the brain is highly questionable (see below).

Ubiquinol (QH_2) is produced in the inner side of the mitochondrial inner membrane as a result of reduction of ubiquinone (Q) by complex I or complex II or electrons donated by cytochrome *b*. Ubiquinol migrates to the outer side of the inner membrane and donates one electron to the iron-sulfur protein (Rieske protein), which then reduces cytochrome c_1, whereas semiquinone ($QH^{\bullet -}$) is formed from ubiquinol at the Q_o site (quinol-oxidizing center) of complex III. Semiquinone is unstable and gives an electron to cytochrome *b* hemes (cytochrome b_L and cytochrome b_H). Electrons from cytochrome *b* are then transferred to the Q_i site. With the first electron, semiquinone is formed at the Q_i site, which is reduced by a second electron to ubiquinol (Fig. 4).

The inhibitors of complex III known so far act on three different sites, and the effect of these inhibitors on ROS generation by mitochondria helps to locate the exact site of superoxide production at complex III.

Stigmatellin inhibits the transfer of electrons from ubiquinol to the iron-sulfur protein, and thus, by preventing the formation of semiquinone, blocks ROS generation induced by antimycin or myxothiazole (92). Antimycin interferes with the transfer of the second electron from cytochrome *b* to the relatively stable semiquinone at the Q_i site; thus, the unstable semiquinone at the Q_o site accumulates, increasing the probability of electron donation to oxygen at the Q_o site. On the other hand, myxothiazole, an inhibitor at the Q_o site, probably prevents semiquinone formation and inhibits superoxide production (28, 98, 99). Myxothiazole inhibited the antimycin-induced H_2O_2 generation in brain mitochondria supported by succinate, indicating that H_2O_2 derives from the Q cycle under this condition (104, 111). Starkov and Fiskum (92), however, demonstrated myxothiazole-induced ROS production in heart and brain mitochondria, which is likely the consequence of another effect of myxothiazole, acting on complex I at the Q site in high concentrations (56). As the Q_o site of complex III is located close to the intermembrane space, superoxide generated at this site by antimycin is less available to the matrix antioxidant enzymes, but could be dismutated by the cytosolic Cu,Zn-SOD. This was clearly indicated by the observation that glutathione depletion did not affect the myxothiazole-sensitive H_2O_2 generation in the presence of succinate and antimycin in brain mitochondria (111).

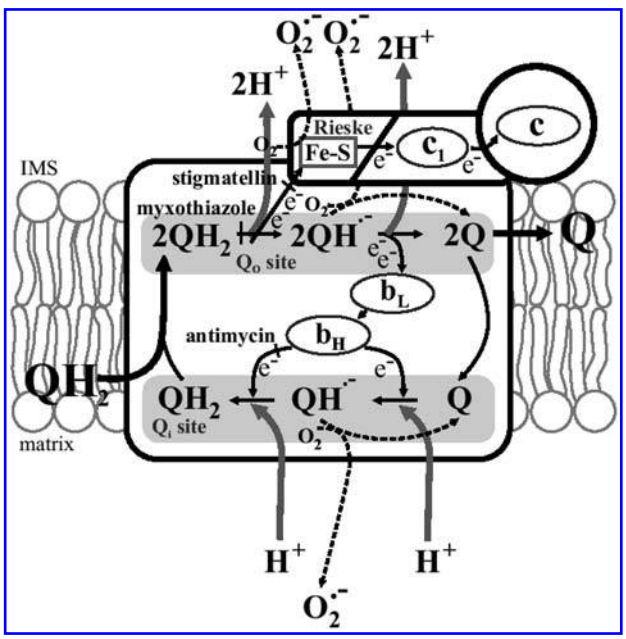

FIG. 4. Possible sites of superoxide ($O_2^{\bullet -}$) generation on complex III. The possibility that superoxide can be produced on the Q_o site or Q_i site is indicated. Abbreviations are as for Fig. 3. The sites of action of complex III inhibitors are shown.

ROS GENERATION BY *IN SITU* MITOCHONDRIA IN NERVE TERMINALS

Results obtained with isolated mitochondria have provided valuable information as to the conditions favoring ROS generation in the respiratory chain, but are not necessarily applicable to *in situ* mitochondria that are present in their normal cytosolic environment. As discussed above, these studies revealed that the site and characteristics of ROS generation in mitochondria are clearly distinctive whether complex I or complex II substrates support mitochondria. However, it is important to address the features of ROS generation in *in situ* mitochondria where substrates derive, in the brain, from the metabolism of glucose, to pass on electrons from both NADH and $FADH_2$ to enter the respiratory chain. The restricted function of the respiratory chain and the resulting increase in ROS generation due to inhibition of the individual respiratory complexes are particularly important in the brain given the numerous reports on impaired function of the respiratory complexes in association with neurodegenerative diseases. Deficiency of complex I was found in postmortem substantia nigra samples from Parkinson's disease patients (87), impaired complexes II and III were described for Huntington's disease (4), and deficiency of complex IV was found in brain samples from Alzheimer's disease patients (49, 73, 78). In particular, the deficiency of complex I is well documented in Parkinson's disease and was linked to the pathogenesis of the disease (21, 70, 85, 86). In addition, inhibition of complex I by the systemic administration of either MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) or rotenone induces the major features of Parkinson's disease (5–7).

Inhibition of respiratory complexes of *in situ* mitochondria was reported to result in an enhanced ROS generation (67, 90), but it was revealed, by detecting in parallel the activity of complexes and the formation of H_2O_2, that different complexes had to be inhibited to different degrees in order to induce excessive ROS production (88). Isolated nerve terminals release a significant amount of H_2O_2 under resting conditions

in glucose-containing medium, indicating that mitochondria *in situ* generate ROS without the respiratory chain being blocked by any added inhibitors (88). When isolated nerve terminals were treated with antimycin, complex III had to be inhibited by >70% before any change occurred in ROS formation; thereafter, a huge increase of H_2O_2 generation was found (88, 89). A similar quantitative relationship between inhibition of complex IV and induction of ROS production was observed in the presence of cyanide (88). Although ROS generation of a large magnitude was induced once complex III or complex IV were inhibited by >70%, given this very substantial threshold for inhibition, it appears to be unlikely that *in vivo* the impaired function of these complexes could be an underlying mechanism in the pathogenesis of neurodegenerative diseases. In contrast, when complex I was inhibited by rotenone to a small degree, generation of H_2O_2 increased significantly (88). The magnitude of ROS formation due to complex I inhibition was always smaller than that observed with the complex III inhibitor antimycin, but it seems to be more important that a small (~16%) inhibition of complex I was already accompanied by an enhancement of ROS formation. ROS produced by this small inhibition was substantial enough to inhibit the endogenous aconitase in the mitochondria (88). In tissue samples from patients dying from Parkinson's disease, the extent of complex I inhibition was characteristically ~25–30% (86, 87). Thus, mitochondria with a partial complex I deficiency found in Parkinson's disease could exhibit an increased tendency to generate oxidative stress. Synaptic mitochondria are especially relevant here because neurodegeneration in Parkinson's disease is likely to start at the nerve terminals as indicated by Betarbet *et al.* (7).

The effect of complex I inhibition in synaptic mitochondria was found to be different from that in mitochondria derived from the whole brain also when respiration and ATP synthesis were studied. In synaptic mitochondria, a relatively small inhibition (~25%) of complex I already impaired respiration and oxidative phosphorylation, whereas in whole brain mitochondria derived mostly from neural cell body and glial cells, oxygen consumption and ATP generation were impaired only after a robust (>70%) complex I inhibition (29, 30). In accordance with this, the membrane potential ($\Delta\Psi_m$) of *in situ* mitochondria in isolated nerve terminals was depolarized at a small degree of complex I inhibition, when the compensatory proton extrusion mechanism by the ATP synthase was prevented (25). These results show that in synaptic mitochondria, complex I has a tight control over respiration, and there is very little reserve proton pumping capacity at this site of the respiratory chain. It was also reported that synaptic mitochondria were able to maintain $\Delta\Psi_m$ when exposed to H_2O_2 even with millimolar concentration, indicating a relative resistance to H_2O_2-induced oxidative stress. However, complex I inhibition to a small degree (~10%) is sufficient to increase substantially the sensitivity of mitochondria to oxidative stress, which was indicated by a gradual collapse of $\Delta\Psi_m$ due to a mild oxidative stress, when complex I was simultaneously inhibited (25). Therefore, results obtained with *in situ* synaptic mitochondria show that a small degree of complex I inhibition already impairs significantly the functional competence of mitochondria, and increases the ROS generation and the sensitivity to oxidative stress, which could be an important factor in the pathogenesis of Parkinson's disease, rendering the nerve terminals a preferential site for cellular deterioration.

ROS GENERATION BY α-KETOGLUTARATE DEHYDROGENASE (α-KGDH)

Recently, the idea that the major site of ROS production in the mitochondria is exclusively the respiratory chain has been challenged by the report that the Krebs cycle enzyme, α-KGDH, is able generate ROS (93, 96). α-KGDH is one of the key enzymes in the Krebs cycle regulated by the $NADH/NAD^+$ ratio, dihydrolipoate/lipoate ratio, Ca^{2+}, and ADP (43, 63, 65, 66, 106). It has been reported that the isolated enzyme produces superoxide and H_2O_2 in the presence of all the cofactors and substrates, except NAD^+ (96). This characteristic was not restricted to the isolated enzyme; the α-KGDH-mediated ROS generation was also demonstrated in isolated brain mitochondria, where out of the respiratory substrates α-ketoglutarate supported the highest rate of H_2O_2 production under conditions of maximum respiration (93). In addition, *in situ* mitochondria in isolated nerve terminals also produced a significantly higher amount of H_2O_2 when α-ketoglutarate, instead of glucose, was the metabolic substrate and the amount of H_2O_2 was sufficient to inhibit the activity of aconitase (96). This enzyme complex is composed of three enzymes: α-KGDH (E1), dihydrolipoamide succinyl transferase (E2), and dihydrolipoamide dehydrogenase (E3). The latter was shown to produce superoxide in a recent study (15) and, in agreement with this, mitochondria from heterozygous knockout mice deficient in dihydrolipoamide dehydrogenase produced significantly less H_2O_2 than mitochondria prepared from their littermate wild-type mice, indicating that the dihydrolipoamide dehydrogenase is most likely responsible for ROS formation by α-KGDH (93). ROS generation by α-KGDH was strongly dependent on the $NADH/NAD^+$ ratio: the higher the $NADH/NAD^+$ ratio, the higher the rate of H_2O_2 formation by the enzyme, whereas the physiological catalytic activity is inhibited (96). This observation strongly supports the suggestion that any condition that restricts the reoxidation of NADH in the respiratory chain thereby increases the intramitochondrial $NADH/NAD^+$ ratio and could promote α-KGDH-mediated ROS production. Inhibition of any component of the respiratory chain or oxidative phosphorylation could favor the ROS generation by the enzyme parallel with an inhibition of the catalytic function. Given that the ROS generation by this enzyme is strongly dependent on the $NADH/NAD^+$ ratio, it is particularly tempting to suggest that α-KGDH could substantially contribute to the oxidative stress due to inhibition of complex I (Fig. 5).

Stimulation of the α-KGDH-mediated ROS formation by Ca^{2+} could explain the earlier observation that Ca^{2+} stimulates mitochondrial ROS production (35, 51, 52), and could be the underlying mechanism by which ROS is generated in neurons following excessive stimulation of *N*-methyl-D-aspartate (NMDA) receptors (8, 34, 55, 83). Excitotoxicity due to excessive NMDA receptor activation involves mitochondrial calcium accumulation (48, 107, 108). α-KGDH-mediated ROS generation could be the link between accumu-

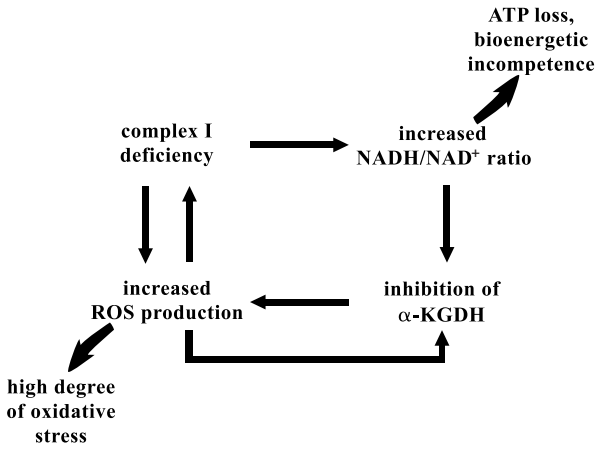

FIG. 5. Vicious cycle due to complex I deficiency involving ROS generation by α-KGDH.

lation of calcium in the mitochondria and neuronal injury induced by NMDA receptor activation. Mitochondrial calcium accumulation is essential for the neuronal damage under this condition; when it is prevented, neurons are protected from injury (14, 94).

α-KGDH could be important in oxidative stress not only as a generator, but also as the key target of ROS in the Krebs cycle. It has been reported that aconitase, succinate dehydrogenase, and α-KGDH are inhibited in the early phase of the H_2O_2-induced oxidative stress in nerve terminals, but the crucial defect is the impaired function of α-KGDH (26, 95), which limits the generation of NADH in the Krebs cycle under this condition (95). In addition, this enzyme has been shown to be glutathionylated by H_2O_2 (75) and inhibited by 4-hydroxynonenal (47), peroxynitrite (77), and β-amyloid (20). The activity of α-KGDH was substantially reduced in postmortem brain samples taken from patients who have suffered from Alzheimer's disease (16, 41, 64) or Parkinson's disease (68, 69). To understand the implications of these observations in the pathogenesis of neurodegeneration needs further investigation. From data available so far, it could be suggested that α-KGDH as a generator and a very sensitive target of oxidative stress could play a critical role in the initiation of neuronal deterioration in the early phase of the disease by limiting the energy production and promoting oxidative stress.

ACKNOWLEDGMENTS

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ABBREVIATIONS

Cu,Zn-SOD, copper/zinc superoxide dismutase; FMN, flavin mononucleotide; H_2O_2, hydrogen peroxide; α-KGDH, α-ketoglutarate dehydrogenase; Mn-SOD, manganese superoxide dismutase; NMDA, *N*-methyl-D-aspartate; ROS, reactive oxygen species; $\Delta\Psi_m$, mitochondrial membrane potential.

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