Reactive oxygen species and mitochondrial diseases

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A variety of diseases have been associated with excessive reactive oxygen species (ROS), which are produced mostly in the mitochondria as byproducts of normal cell respiration. The interrelationship between ROS and mitochondria suggests shared pathogenic mechanisms in mitochondrial and ROS-related diseases. Defects in oxidative phosphorylation can increase ROS production, whereas ROS-mediated damage to biomolecules can have direct effects on the components of the electron transport system. Here, we review the molecular mechanisms of ROS production and damage, as well as the existing evidence of mitochondrial ROS involvement in human diseases.

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Molecular mechanisms of mitochondrial ROS production

Mitochondria are unique organelles, as they are the main site of oxygen metabolism, accounting for approximately 85–90% of the oxygen consumed by the cell.\textsuperscript{1,2} Incomplete processing of oxygen and/or release of free electrons results in the production of oxygen radicals. Mitochondria constantly metabolize oxygen thereby producing reactive oxygen species (ROS) as a byproduct. These organelles have their own ROS scavenging mechanisms that are required for cell survival.\textsuperscript{3} It has been shown, however, that mitochondria produce ROS at a rate higher than their scavenging capacity, resulting in the incomplete metabolism of approximately 1–3% of the consumed oxygen.\textsuperscript{4,5} The byproducts of incomplete oxygen metabolism are superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radical (OH•). The formation of superoxide occurs via the transfer of a free electron to molecular oxygen. This reaction occurs at specific sites of the electron transport chain (ETC), which resides in the inner mitochondrial membrane [Figure 1(a)]. ETC complexes I (NADH dehydrogenase) and III (ubisemiquinone) produce most of the superoxide,\textsuperscript{4,6,7} which is then scavenged by the mitochondrial enzyme manganese superoxide dismutase (MnSOD) to produce H$_2$O$_2$. Since mitochondria do not contain catalase, their only defense against the potentially toxic properties of H$_2$O$_2$ is the enzyme glutathione peroxidase (GSPx). GSPx requires reduced glutathione (GSH) as a coenzyme and converts H$_2$O$_2$ to water, thus completely detoxifying ROS. However, in the presence of reduced transition metals, H$_2$O$_2$ can produce the highly reactive OH•, which can cause extensive damage to DNA, proteins, and lipids [Figure 1(a)].\textsuperscript{8}

Two other important radical species are nitric oxide (NO) and peroxynitrite (ONOO–). Recent evidence has demonstrated that mitochondria possess their own nitric oxide synthase (mtNOS)\textsuperscript{9} and can produce endogenous NO and ONOO–.\textsuperscript{10,11} Although the main source of ONOO– is through the reaction of NO with superoxide [Figure 1(a)], there are also several other ways to produce ONOO– in a cell.\textsuperscript{12–16} Mitochondrial NO decays mainly via ONOO– formation, ubiquinol oxidation, and reversible binding to cytochrome c oxidase.\textsuperscript{17}

ROS-mediated damage

Under normal conditions, the effects of ROS are counteracted by a variety of antioxidants, by both enzymatic and nonenzymatic mechanisms. Oxidative
Figure 1. Generators and targets of reactive oxygen species (ROS) in mitochondria.
(a) A schematic diagram of the generation of the main mitochondrial reactive oxygen species and their targets. Solid arrows with solid arrowheads (▶) indicate generation of molecules. Solid arrows with pointed arrowheads (→) indicate diffusion of molecules. Hatched arrows indicate damaging effects.
(b) An illustration of the ‘vicious cycle’ hypothesis. MtDNA damage leads to defective ETC complexes, which consequently increase ROS production, potentially leading to further mtDNA damage.
stress is considered to be the result of an imbalance of two opposing and antagonistic forces, ROS and antioxidants, in which the effects of ROS are more potent than the compensatory capacity of antioxidants. In the case of mitochondrial-derived ROS, superoxide is the first radical produced. It is a highly reactive species and does not diffuse easily throughout the cell. Because the main site of $O_2^-$ production is the inner mitochondrial membrane, the mitochondrial DNA (mtDNA) has been hypothesized to be a major target for ROS damage [Figure 1(b)]. A circumstantial correlation between ROS and mtDNA damage was shown by measuring the levels of mtDNA rearrangements in heart and skeletal muscle tissue of mice. The level of mtDNA rearrangements were higher in the heart, which coincides with the fact that heart has lower antioxidant defenses than skeletal muscle. $H_2O_2$ is the next key player in mitochondrially derived ROS, as it is the product of superoxide detoxification by MnSOD. $H_2O_2$ is not a free radical by definition because it lacks free electrons. Nevertheless, its role in ROS-mediated damage is extremely significant by virtue of chemical versatility and diffusibility. $H_2O_2$ is a substrate in many physiological and abnormal chemical reactions both intracellularly and extracellularly. Due to its small size and relatively benign reactivity, compared to the rest of the ROS, $H_2O_2$ can diffuse freely across several cell radii; therefore, it is able to mediate toxic effects far from the site of ROS production. Although by itself $H_2O_2$ is not a major source of oxidative damage, it can react with free transition metals via the Fenton reaction ($Fe^{2+} + H_2O_2 \leftrightarrow Fe^{3+} + OH + OH\bullet$), producing the extremely reactive hydroxyl radical. $OH\bullet$ has a very short half-life and reacts with virtually any molecules in close proximity [Figure 1(a)]. There is no known scavenger for $OH\bullet$, but $OH\bullet$ toxicity can be avoided by minimizing the levels of $H_2O_2$ and most importantly the availability of free transition metals (e.g. $Fe^{2+}$, $Cu^{+}$).

Nitric oxide has also been implicated in ROS-mediated damage. NO has a dual personality, which is both beneficial, and detrimental. In some instances, different intracellular levels of NO can mediate diverse effects. For example, physiological levels of NO inhibit the opening of the mitochondrial permeability transition pore (PTP), whereas high NO concentrations promote PTP opening. In this review, however, we will only discuss the harmful effects of NO. High levels of NO are cytotoxic, although the exact mechanism associated with this effect is still unclear. It may be involved in inflammatory, neurodegenerative, and cardiovascular pathological processes. NO can regulate aerobic respiration by reversible inhibition of cytochrome c oxidase. Interestingly, because of the short half-life and diffusibility of NO, any cell that produces excess NO will inhibit its own respiration and respiration of surrounding cells, which may contribute to the cytotoxic effects of NO. For example, in co-cultures of astrocytic and neuronal cells, astrocytic-derived NO causes both reversible and irreversible damage to the neuronal electron transport chain. Peroxynitrite is a highly damaging agent with a vast repertoire of targets and detrimental cellular effects. $ONOO^-$ modifies proteins by nitrating tyrosine residues, forming dityrosine, and oxidizing tryptophan and cysteine. The main mitochondrial targets of peroxynitrite are complexes I, II, IV and V, aconitase, creatine kinase, superoxide dismutase, mitochondrial membranes, and mtDNA. Damage of these molecules may induce mitochondrial swelling, depolarization, calcium release, and permeability transition. Uptake of calcium by mitochondria induces mtNOS which increases $ONOO^-$ levels in the organelle, leading to release of cytochrome c, increase of lipid peroxidation and to a subsequent release of calcium in the cytosol, a process which has been suggested to be a feedback loop preventing mitochondrial calcium overload. Another important finding is that $ONOO^-$ inhibits MnSOD enzymatic activity by nitration and oxidation of critical tyrosine residues. Curiously, $ONOO^-$ seems to preferentially damage certain proteins in a cell-type specific manner. The molecule affects the electron transport chain of neurons, but not astrocytes, however, it preferentially depletes glutathione (GSH) in astrocytes.

**Mitochondrial dysfunction and ROS in human pathology**

There has been a great deal of research on the role of ROS in the pathogenesis of a number of human diseases. A multitude of theories exist, attempting to explain the mechanisms of ROS-mediated damage and its effects in the progression of diseases. In many diseases, primary mitochondrial involvement is profound and evident. In others, the mitochondrial participation is only suspected.
Mitochondrial diseases

In the last 15 years, several clinical syndromes were associated with mtDNA mutations, the most common being NARP (neurogenic muscle weakness, ataxia and retinitis pigmentosa), MELAS (mitochondrial encephalomyopathy lactic acidosis, and stroke-like episodes), MERRF (myoclonic epilepsy and ragged-red fibers), LHON (Leber hereditary optic neuropathy), and KSS (Kearns-Sayre syndrome, ophthalmoplegia, ataxia, retinitis pigmentosa, cardiac conduction defect and elevated cerebrospinal fluid protein) (see article by DiMauro in this issue). MtDNA encodes few proteins which are involved in the electron transport chain, the main source of ROS in cells. This special situation highlights the theory of the ‘vicious cycle’, a theory attractive within the realm of degenerative processes. In this cycle, an inherited or random primary mitochondrial mutation initially induces a respiratory defect, that increases the leakage of ROS from the electron transport chain. Subsequently, ROS may trigger accumulation of secondary mtDNA mutations exacerbating mitochondrial respiratory defects and consequently increasing production of ROS and lipid peroxides from mitochondria.

It has been demonstrated that after exposing cells to oxidative stress, mtDNA damage is more extensive and persists longer than damage in nuclear DNA (nDNA). Several reasons may contribute to this selective vulnerability: (i) mtDNA lacks histones which are protective against free radical damage, (ii) mtDNA lacks an adequate repair system, rendering it unable to cope with extensive damage, especially strand breaks, (iii) mtDNA has very few non-coding sequences, therefore increasing the likelihood of a DNA alteration to affect a gene, and (iv) mtDNA is located near the inner mitochondrial membrane, a major site of oxygen radical production. However, a correlation between defective oxidative phosphorylation and increased rate of accumulation of mtDNA deletions has not been observed in patients with mitochondrial disorders. Using a specific PCR approach to detect the so-called mtDNA ‘common deletion’ and long PCR to detect a plethora of mtDNA rearrangements, Tengan et al. could not find an increase in mtDNA deletions in muscle of patients with pathogenic mtDNA point mutations. This observation argues against the ‘vicious cycle theory’, as one would expect that the ETC defect caused by the mtDNA point mutations should lead to an increase in ROS, and consequently an increase in mtDNA rearrangements. Therefore, the ‘vicious cycle’ hypothesis [Figure 1(b)] continues to be an attractive but unproven hypothesis.

Several studies have demonstrated that mtDNA mutations associated with human disease lead to ETC complex dysfunction, increased production of ROS, and oxidative damage, as is the situation in MELAS, where hydroxyl radical damage to mtDNA can be accelerated by a specific mitochondrial genotype associated with the disease. In MELAS and MERRF patients, the intracellular levels of hydrogen peroxide and oxidative damage to DNA and lipids were increased in skin fibroblasts. In whole blood cells of patients with MELAS-related mitochondriopathy and patients with LHON, ROS-associated telomere shortening was observed. Finally in a mouse model of sarcopenia, mtDNA deletion mutations in muscle fibers co-localized with increased levels of oxidative damage to nucleic acids. Furthermore, ROS-mediated damage selectively to mtDNA has been reported in other diseases, like Down syndrome, acute pancreatitis, multiple sclerosis, end-stage renal disease, and left ventricular remodeling and failure following myocardial infarction.

As discussed above, most mitochondrial ROS are formed at complexes I and III. Many clinical syndromes have been associated with isolated complex I deficiencies have been described, including fatal infantile lactic acidosis, adult-onset exercise intolerance, focal dystonia, LHON, cardiomyopathy with cataracts, hepatopathy with tubulopathy, Leigh’s disease, cataracts and developmental delay, and lactic academia in the neonatal period followed by mild symptoms. It is unclear at this point if some of these symptoms are exacerbated by increased ROS production, even though complex I impairment leads to increased ROS production in submitochondrial particles and cell systems. In studies of human xenomitochondrial
cybrids harboring a 40% complex I deficiency and a drug-induced model of complex I inhibition,\(^{49,50}\) correlations between the levels of complex I impairment and cell respiration, cell growth, ROS production, lipid peroxidation, mitochondrial membrane potential, and apoptosis were observed. Interestingly, cell death was quantitatively associated with ROS production, rather than with complex I deficiency.\(^{51}\) This suggests that partial ETC defects may, in some instances, mediate cellular damage by ROS-related processes.

It has been shown that complex I impairment induces MnSOD and/or increases ROS production.\(^{45,46,52}\) Therefore, a complex I defect may not be accompanied by increased ROS, if MnSOD is elevated. However, MnSOD as the only scavenger might not be sufficient to prevent oxidative damage. Its action need to be coupled with GSPx/GSH detoxification of \(\text{H}_2\text{O}_2\), in order to avoid the formation of the highly reactive \(\text{OH}^\bullet\), as observed in cells from a patient with cardiomyopathy with cataracts.\(^{45}\)

Fewer studies have been described for complex III deficiencies. Mutations in the apocytochrome \(b\) gene have been described in patients with myopathies\(^{53}\) but also with encephalopathies.\(^{54}\) In transmitochondrial cybrids containing mtDNA from a patient with Parkinsonism and MELAS, high levels of a mtDNA with a mutation in the apocytochrome \(b\) gene were associated with respiratory deficiency and complex III defect. This deficiency was also associated with increased hydrogen peroxide production.\(^{55}\) Such an increase in the ROS levels in the central nervous systems could explain the Parkinsonism in this patient.

**Neurodegenerative diseases**

Increasingly, research efforts are investigating the involvement of ROS and mitochondria in the pathogenesis of common neurodegenerative diseases: Parkinson disease (PD), Alzheimer’s disease (AD), amyotrophic lateral sclerosis (ALS), Huntington’s disease (HD), and Friedreich’s ataxia (FA). Although an extensive review of research efforts in this field would be beyond the scope of this paper, we will briefly discuss the mitochondrial and ROS involvement in these conditions. Increased ROS production in neurodegenerative processes may affect normal mitochondrial parameters like ATP production, membrane potential, permeability transition pore activation, and calcium uptake. These changes can lead to neuronal death, mainly through excitotoxic pathways, involving oxidation of macromolecules and apoptosis.

More than a decade ago, the first strong evidence of mitochondrial involvement in the pathogenesis of a neurodegenerative disease came to light when complex I deficiency was identified in substantia nigra\(^{56}\) and platelet mitochondria of Parkinson disease patients.\(^{57}\) Subsequently, enzymatic deficiencies in the electron transport chain were identified in additional neurodegenerative diseases: complex IV deficiency in AD and ALS,\(^{58–60}\) and complex II and III in HD and FA.\(^{61}\) The common denominator of mitochondrial dysfunction in these diseases led to hypotheses related to mtDNA involvement. In HD and FA the genetic defects appear to be in nuclear genes that encode non-respiratory proteins (huntingtin for HD, and frataxin for FA). This fact suggests that the observed respiratory deficiencies are secondary to the pathogenic initiating factors. For PD and AD the picture is unclear. Some reports showed that a proportion of patients, have evidence of mtDNA abnormalities as a key player in the progress of disease.\(^{62}\) Additional evidence of an association between ROS and mitochondrial dysfunction comes from the observation that mutations in the SOD1 gene can cause ALS.\(^{63}\) Also, mitochondrial abnormalities are seen early in the development of the disease.\(^{60}\) However, it is still unclear if the mutated SOD1 alters ROS production, or even whether the mitochondrial abnormalities are secondary to a cell death stimulus.

Oxidative damage is also a common finding in all neurodegenerative diseases. Evidence of oxidative damage in PD,\(^{68,69,64–67}\) AD,\(^{68–70}\) ALS,\(^{71–74}\) HD,\(^ {75–77}\) and FA\(^ {78,79}\) strengthened the link between abnormal mitochondrial function, increased ROS production, and neurodegeneration. Recent interesting evidence suggest that motor neurons are particularly susceptible to damage from endogenously produced ONOO\(^{-}\),\(^ {80,81}\) and protein bound nitrotyrosine has been observed in motor neurons from human ALS patients and tissue of a mouse model harboring a transgene coding for a mutated form of SOD1 found in some patients (G93A-SOD1).\(^ {82}\) An interesting hypothesis is that the high polyunsaturate level of brain mitochondrial phospholipids might predispose them to peroxidation, hence brain mitochondria may be more susceptible to neurodegenerative disorders that progress via mechanisms of mitochondrial oxidative damage.\(^ {83}\)
Aging

Although it is doubtful that aging is a disease, mitochondrial ROS involvement in this process is strongly suggested by experimental data. A prevalent theory for aging is a variant of the vicious cycle concept we described previously. MtDNA is constantly exposed to ROS generated by the mitochondrial electron transport chain, and mutations may accumulate exponentially with age. The simultaneous increase in lipid peroxidation and oxidation of mitochondrial proteins adds to the oxidative stress effects, initiating the vicious cycle of molecular degeneration. This putative vicious cycle can operate at different rates in various tissues, leading to differential accumulation of oxidative damage, which could explain the differences in functional impairment and deterioration of different tissues in the aging process.

There is substantial evidence that damage to mtDNA accumulates with age. Among them, the most significant is the 10-fold increase in an oxidative damage marker (8-hydroxy-2-deoxyguanosine) in mtDNA versus nDNA from human brain. 84 A study of aging in rhesus monkeys revealed that there are significant decreases with increasing age in the activities of complex I and IV, as well as mitochondrial ATP generation. 85 Finally, several mtDNA point mutations also increase with normal aging. 86–89 What is not clear at this point, is whether such mutations are generated by ROS-mediated damage.

Concluding remarks

Clearly, our understanding of the intricate relationship between mitochondrial function, ROS production, ROS damage and the development of a clinical phenotype is still very limited. Even the link between ROS and mtDNA mutations is still a matter of controversy, lacking experimental evidence and relying mostly on hypothetical models. This is particularly evident for mtDNA deletions, a widely accepted marker of aging. The origin of such mtDNA rearrangements is unclear, and oxidative damage does not provide an adequate model for the generation of mtDNA deletions. In addition, the role of ROS and mitochondrial in neurodegenerative disorders and aging is also a matter of constant debate, as increased oxidative damage may be a consequence rather than the cause of the disease process. Because of the growing interest in this area, the next few years should bring answers to many of these questions.

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