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# Topical Review

# Mitochondrial formation of reactive oxygen species

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The reduction of oxygen to water proceeds via one electron at a time. In the mitochondrial respiratory chain, Complex IV (cytochrome oxidase) retains all partially reduced intermediates until full reduction is achieved. Other redox centres in the electron transport chain, however, may leak electrons to oxygen, partially reducing this molecule to superoxide anion ($O_2^{-}\bullet$). Even though $O_2^{-}\bullet$ is not a strong oxidant, it is a precursor of most other reactive oxygen species, and it also becomes involved in the propagation of oxidative chain reactions. Despite the presence of various antioxidant defences, the mitochondrion appears to be the main intracellular source of these oxidants. This review describes the main mitochondrial sources of reactive species and the antioxidant defences that evolved to prevent oxidative damage in all the mitochondrial compartments. We also discuss various physiological and pathological scenarios resulting from an increased steady state concentration of mitochondrial oxidants.

## The chemistry of oxygen and oxidative stress

Reactive oxygen species (ROS) is a phrase used to describe a variety of molecules and free radicals (chemical species with one unpaired electron) derived from molecular oxygen. Molecular oxygen in the ground state is a bi-radical, containing two unpaired electrons in the outer shell (also known as a triplet state). Since the two single electrons have the same spin, oxygen can only react with one electron at a time and therefore it is not very reactive with the two electrons in a chemical bond. On the other hand, if one of the two unpaired electrons is excited and changes its spin, the resulting species (known as singlet oxygen) becomes a powerful oxidant as the two electrons with opposing spins can quickly react with other pairs of electrons, especially double bonds.

The reduction of oxygen by one electron at a time produces relatively stable intermediates. Superoxide anion ($O_2^{-}\bullet$), the product of a one-electron reduction of oxygen, is the precursor of most ROS and a mediator in oxidative chain reactions. Dismutation of $O_2^{-}\bullet$ (either spontaneously or through a reaction catalysed by superoxide dismutases) produces hydrogen peroxide ($H_2O_2$), which in turn may be fully reduced to water or partially reduced to hydroxyl radical ($OH\bullet$), one of the strongest oxidants in nature. The formation of $OH\bullet$ is catalysed by reduced transition metals, which in turn may be re-reduced by $O_2^{-}\bullet$, propagating this process (Liochev & Fridovich, 1999). In addition, $O_2^{-}\bullet$ may react with other radicals including

nitric oxide ($NO\bullet$) in a reaction controlled by the rate of diffusion of both radicals. The product, peroxynitrite, is also a very powerful oxidant (Beckman & Koppenol, 1996; Radi *et al.* 2002*b*; Fig. 1). The oxidants derived from $NO\bullet$ have been recently called reactive nitrogen species (RNS).

'Oxidative stress' is an expression used to describe various deleterious processes resulting from an imbalance between the excessive formation of ROS and/or RNS and limited antioxidant defences (Fig. 1). Whilst small fluctuations in the steady-state concentration of these oxidants may actually play a role in intracellular signalling (Droge, 2002), uncontrolled increases in the steady-state concentrations of these oxidants lead to free radical-mediated chain reactions which indiscriminately target proteins (Stadtman & Levine, 2000), lipids (Rubbo *et al.* 1994), polysaccharides (Kaur & Halliwell, 1994) and DNA (Richter *et al.* 1988; LeDoux *et al.* 1999).

*In vivo*, $O_2^{-}\bullet$ is produced both enzymatically and non-enzymatically. Enzymatic sources include NADPH oxidases located on the cell membrane of polymorphonuclear cells, macrophages and endothelial cells (Babior, 2000; Vignais, 2002; Babior *et al.* 2002) and cytochrome $P_{450}$-dependent oxygenases (Coon *et al.* 1992). The proteolytic conversion of xanthine dehydrogenase to xanthine oxidase provides another enzymatic source of both $O_2^{-}\bullet$ and $H_2O_2$ (and therefore constitutes a source of $OH\bullet$) and has been proposed to mediate deleterious processes *in vivo* (Yokoyama *et al.* 1990).

**Table 1. Compartmental localization of the main mitochondrial sources of superoxide anion**

| Component | Localization | References |
|---|---|---|
| Complex I (NADH dehydrogenase) | Inner membrane/ inner side | (Turrens & Boveris, 1980; Turrens *et al.* 1982; Genova *et al.* 2001; Kushnareva *et al.* 2002) |
| Complex II (succinate dehydrogenase) | Inner membrane/ inner side | (Zhang *et al.* 1998; Lenaz, 2001) |
| Complex III (ubiquinol–cytochrome *c* reductase) | Inner membrane/ inner side | (Boveris *et al.* 1976; Cadenas *et al.* 1977; Turrens *et al.* 1985) |
| Complex III (ubiquinol–cytochrome *c* reductase) | Inner membrane/ outer side | (Han *et al.* 2001; Starkov & Fiskum, 2001) |
| External NADH dehydrogenase (yeast) | Inner membrane/ outer side | (Fang & Beattie, 2003) |
| Glycerolphosphate dehydrogenase | Inner membrane/ outer side | (Drahota *et al.* 2002) |
| Dehydroorotate dehydrogenase | Matrix | (Forman & Kennedy, 1976) |
| Mono amino oxidase | Outer membrane/ inner side | (Hauptmann *et al.* 1996; Cadenas & Davies, 2000) |

The non-enzymatic production of $O_2^-\cdot$ occurs when a single electron is directly transferred to oxygen by reduced coenzymes or prosthetic groups (for example, flavins or iron sulfur clusters) or by xenobiotics previously reduced by certain enzymes (for example, the anticancer agent adriamycin or the herbicide paraquat). The mitochondrial electron transport chain contains several redox centres that may leak electrons to oxygen, constituting the primary source of $O_2^-\cdot$ in most tissues.

## Detection of ROS and RNS

The formation of ROS and RNS may be monitored using a variety of procedures including fluorometric and spectrophotometric methods, chemiluminescence and electron paramagnetic resonance (Chance *et al.* 1979; Pou *et al.* 1989; Tarpey & Fridovich, 2001).

Many of these methods rely on the redox properties of specific ROS or RNS, and therefore are prone to artifacts caused by species of similar reactivity or by reactive intermediates produced by the probe itself (Picker & Fridovich, 1984; Faulkner & Fridovich, 1993; Liochev & Fridovich, 1995, 1998).

Specific inhibitory enzymes may be added to unequivocally identify the species (for example, superoxide dismutase or catalase to eliminate $O_2^-\cdot$ or $H_2O_2$, respectively) but these enzymes do not determine whether these ROS are the primary species or just intermediates formed in the detection reaction. For example, a research group concluded that $O_2^-\cdot$ was produced by the enzyme glucose oxidase because superoxide dismutase inhibited the reduction of nitroblue tetrazolium in the presence of glucose, when in reality the probe was reacting with the enzyme's prosthetic group and $O_2^-\cdot$ was formed after this reaction (Al-Bekairi *et al.* 1994; Liochev & Fridovich, 1995). Similarly, luminol and lucigenin may also produce $O_2^-\cdot$ during their oxidation, leading to the false conclusion that $O_2^-\cdot$ was actually formed in the process (Liochev & Fridovich, 1998). When possible, it is recommended to use
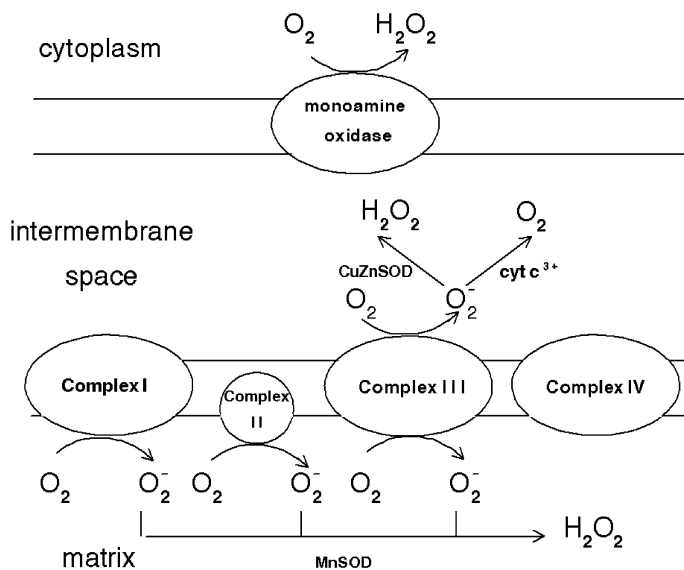


**Figure 1. Sites of superoxide formation in the respiratory chain**

Various respiratory complexes leak electrons to oxygen producing primarily superoxide anion ($O_2^-\cdot$). This species may reduce cytochrome *c* (in the intermembrane space), or may be converted to hydrogen peroxide ($H_2O_2$) and oxygen (in both the matrix and the intermembrane space). Increased steady state concentrations of $O_2^-\cdot$ may reduce transition metals (which in turn react with $H_2O_2$ producing hydroxyl radicals ($OH\cdot$)) or may react with nitric oxide to form peroxynitrite. Both $OH\cdot$ and peroxynitrite are strong oxidants which indiscriminately react with nucleic acids lipids and proteins.

more than one method to identify specific ROS, particularly if they are based on different properties of a given species. For example, $O_2^-\cdot$ may oxidize certain probes (adrenaline (epinephrine) and hydroethidine) and also reduce others (cytochrome *c* or nitroblue tetrazolium; Butler *et al.* 1975). The choice of methods may also vary depending on the source of ROS under study. For example, if one is monitoring $O_2^-\cdot$ by the respiratory chain, cytochrome *c* and nitroblue tetrazolium are usually not good choices because they react with other carriers in the electron transport chain.

## Mitochondrial sources of superoxide and nitric oxide

The standard reduction potential for the conversion of molecular oxygen to $O_2^-\cdot$ is −0.160 V (Wood, 1987). The respiratory chain includes a variety of redox centres with standard reduction potentials between −0.32 V (NAD(P)H) and +0.39 V (cytochrome $a_3$ in Complex IV). Given the highly reducing intramitochondrial environment, various respiratory components, including flavoproteins, iron–sulfur clusters and ubisemiquinone, are thermo-dynamically capable of transferring one electron to oxygen. Moreover, most steps in the respiratory chain involve single-electron reactions, further favouring the monovalent reduction of oxygen. On the other hand, the mitochondrion possesses various antioxidant defences designed to eliminate both $O_2^-\cdot$ and $H_2O_2$ (see below). As a result, the steady state concentrations of $O_2^-\cdot$ and $H_2O_2$ have been estimated to be around $10^{-10}$ M and $5 \times 10^{-9}$ M, respectively (Cadenas & Davies, 2000).

Over the past 35 years several laboratories have identified a variety of mitochondrial sources of $O_2^-\cdot$ including several respiratory complexes and individual enzymes. Superoxide formation occurs on the outer mitochondrial membrane, in the matrix and on both sides of the inner mitochondrial membrane (Table 1, Fig. 2). Whilst the $O_2^-\cdot$ generated in the matrix is eliminated in that compartment, part of the

$O_2^-\cdot$ produced in the intermembrane space may be carried to the cytoplasm via voltage-dependent anion channels (Han *et al.* 2003).

The relative contribution of every site to the overall $O_2^-\cdot$ production varies from organ to organ and also depends on whether mitochondria are actively respiring (State 3) or the respiratory chain is highly reduced (State 4) (Barja, 1999). Although Complex III appears to be responsible for most of the $O_2^-\cdot$ produced in heart and lung mitochondria (Turrens & Boveris, 1980; Turrens *et al.* 1982), $O_2^-\cdot$ formation by Complex I appears to be the primary source of $O_2^-\cdot$ in the brain under normal conditions (Barja & Herrero, 1998; Barja, 1999). Moreover, Complex I is the primary source of ROS in a variety of pathological scenarios ranging from ageing to Parkinson's disease (see below) (Barja & Herrero, 1998; Betarbet *et al.* 2002; Kushnareva *et al.* 2002; Nicholls, 2002; Sherer *et al.* 2003*a,b*;Trojanowski, 2003).

The rate of $O_2^-\cdot$ formation by the respiratory chain is controlled primarily by mass action, increasing both when electron flow slows down (increasing the concentration of electron donors, R•) and when the concentration of oxygen increases (eqn (1); Turrens *et al.* 1982).

$$\frac{d[O_2]}{dt} = k\,[O_2]\,[R\cdot]. \qquad (1)$$

The energy released as electrons flow through the respiratory chain is converted into a $H^+$ gradient through the inner mitochondrial membrane (Mitchell, 1977). This gradient, in turn, dissipates through the ATP synthase complex (Complex V) and is responsible for the turning of a rotor-like protein complex required for ATP synthesis (Noji & Yoshida, 2001). In the absence of ADP, the movement of $H^+$ through ATP synthase ceases and the $H^+$ gradient builds up causing electron flow to slow down and the respiratory chain to become more reduced (State IV respiration). As a result, the physiological steady state

**Figure 2. Mechanism of electron flow in the Q-cycle in Complex III**

Ubiquinone is reduced either by Complex I or II or by electrons transferred from cytochrome *b* on the inner side of the membrane (i), producing ubiquinol ($QH_2$). The reduced form transfers one electron to oxygen via the Rieske iron–sulfur protein (ISP), cytochrome $c_1$ and cytochrome *c*. The terminal oxidase is reduced by four electrons (two by copper atoms and two by cytochromes) which in turn are transferred to oxygen producing water. Cytochrome *b* is reduced by the ubisemiquinone formed on the outer side ($Q\cdot_{(o)}$), but this species cannot be formed unless an electron is previously transferred by $QH_2$ to ISP. Superoxide may be produced on both sides of the inner membrane via the autoxidation of Q but the contribution of each pool has not yet been determined.
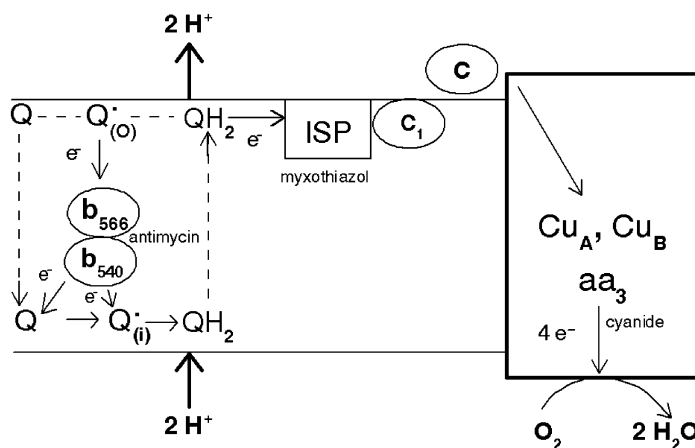
concentration of $O_2^-\bullet$ formation increases (Boveris *et al.* 1972). The formation of $O_2^-\bullet$ may be further increased in the presence of certain inhibitors (for example rotenone, which inhibits Complex I, or antimycin, an inhibitor of Complex III), which cause those carriers upstream from the site of inhibition to become fully reduced. In Complex I, the primary source of $O_2^-\bullet$ appears to be one of the iron–sulfur clusters (either N-1$\alpha$ or N-2; Genova *et al.* 2001; Kushnareva *et al.* 2002). In Complex III, most of the $O_2^-\bullet$ appears to be formed as a result of the autoxidation of ubisemiquinone both on the outer and inner sides of the inner mitochondrial membrane (Table 1).

Although $O_2^-\bullet$ production increases as the respiratory chain becomes more reduced, not all mitochondrial inhibitors have this effect. Most of the production of $O_2^-\bullet$ by Complex III is actually inhibited if electron flow between the Rieske Fe–S protein and oxygen is blocked (for example, by myxothiazol, cyanide or cytochrome *c* depletion; Fig. 2; Turrens *et al.* 1985). This inhibitory effect indicates that $O_2^-\bullet$ must be produced as a result of the autoxidation of ubisemiquinone (Q•), an intermediate produced in Complex III during the Q-cycle (Trumpower, 1990; Fig. 2). The first experimental evidence for the Q-cycle came in 1974, when Chance and co-workers reported that addition of oxygen to anaerobic mitochondria caused a transient reduction of cytochrome *b*, instead of the expected oxidation. This puzzling result became known as the 'oxidant-induced reduction of cytochrome *b*'. According to this model, coenzyme Q is fully reduced in the inner side of the mitochondrial membrane (ubiquinol, $QH_2$) and then migrates to the outer side of the inner membrane carrying 2 $H^+$ that become part of the pool needed to sustain ADP phosphorylation. Once on the outer side of the membrane, one electron is transferred to cytochrome $c_1$ (via the Rieske Fe–S protein), resulting in the formation of Q•. The second electron is needed to reduce cytochrome *b*, but eventually some electrons leak to oxygen, producing $O_2^-\bullet$. Under normal conditions, since cytochrome *b* cannot be reduced unless an electron is transferred to oxygen, an oxidant is needed for the reduction of this cytochrome. As a result of this process, antimycin, by binding to cytochrome *b*, increases the steady state concentration of Q•, also increasing $O_2^-\bullet$ formation (Fig. 2). Myxothiazol (by inhibiting the Rieske Fe–S protein) and cyanide or cytochrome *c* depletion (by preventing the electrons from reaching molecular oxygen), inhibit the increased $O_2^-\bullet$ production observed in the presence of antimycin (Turrens *et al.* 1985). Yet, the inhibition by myxothiazol is not complete, suggesting that there are additional sites in Complex III responsible for $O_2^-\bullet$ formation (Starkov & Fiskum, 2001; Young *et al.* 2002; Fig. 2).

The simultaneous formation of $O_2^-\bullet$ and nitric oxide produces peroxynitrite, a very strong oxidant and nitrating agent. Nitric oxide is a vasodilator resulting from the breakdown of arginine to citrulline, in a reaction catalysed by a family of NADPH-dependent enzymes called nitric oxide synthases. Two separate laboratories have recently discovered that the mitochondrial matrix contains a unique form of nitric oxide synthase (Ghafourifar & Richter, 1997; Giulivi *et al.* 1998; Alvarez *et al.* 2003). Although its physiological role is still unclear, the formation of nitric oxide in mitochondria may have important consequences because this compound binds to haem groups from cytochromes (in particular cytochrome oxidase) and inhibits respiration (Poderoso *et al.* 1996) This may, in turn, stimulate $O_2^-\bullet$ formation (for example from Complex I; Poderoso *et al.* 1996), which in turn may react with more nitric oxide forming peroxynitrite, an oxidant capable of inhibiting important enzymes and affecting mitochondrial integrity (Cassina & Radi, 1996; Radi *et al.* 2002*a*).

Since NO• formation requires oxygen, the rate at which it is produced varies with the intramitochondrial oxygen concentration (Alvarez *et al.* 2003). Assuming an intra-mitochondrial oxygen concentration of 20 $\mu$M, Alvarez *et al.* recently estimated that the intramitochondrial steady-state concentrations of NO• and peroxynitrite in the liver are around 36 nM and 2.2 nM, respectively (Alvarez *et al.* 2003). Other investigators (Wittenberg & Wittenberg, 1989) have estimated that the intramitochondrial oxygen concentration is considerably lower (3 $\mu$M), which would imply lower NO• and peroxynitrite steady-state concentrations.

## Mitochondrial antioxidant defences

The deleterious effects resulting from the formation of ROS in the mitochondrion are, to a large extent, prevented by various antioxidant systems. Superoxide is enzymatically converted to $H_2O_2$ by a family of metalloenzymes called superoxide dismutases (SOD; Fridovich, 1995). Since $O_2^-\bullet$ may either reduce transition metals, which in turn can react with $H_2O_2$ producing OH• or spontaneously react with NO• to produce peroxynitrite, it is important to maintain the steady-state concentration of $O_2^-\bullet$ at the lowest possible level. Thus, although the dismutation of $O_2^-\bullet$ to $H_2O_2$ and $O_2$ can also occur spontaneously, the role of SODs is to increase the rate of the reaction to that of a diffusion-controlled process.

The mitochondrial matrix contains a specific form of SOD, with manganese in the active site (Fridovich, 1995), which eliminates the $O_2^-\bullet$ formed in the matrix or on the inner side of the inner membrane. The expression of MnSOD is further induced by agents that cause oxidative stress, including radiation and hyperoxia, in a process mediated by the oxidative activation of the nuclear transcription factor NF$\kappa$B (Oberley *et al.* 1987; Das *et al.* 1995; Warner *et al.* 1996; Tsan *et al.* 2001; Murley *et al.* 2001).

The steady-state concentration of $O_2^-\cdot$ in the intermembrane space is controlled by three different mechanisms. Firstly, this compartment contains a different SOD isozyme which contains copper and zinc instead of manganese (CuZnSOD (Okado-Matsumoto & Fridovich, 2001)) and is also found in the cytoplasm of eukaryotic cells. Secondly, the intermembrane space contains cytochrome *c* which can be reduced by $O_2^-\cdot$ ($k = 10^7$ M$^{-1}$ s$^{-1}$, Butler *et al.* 1975) regenerating oxygen in the process (Fig. 2). The reduced cytochrome *c* can then transfer electrons to the terminal oxidase. Thus, some of the electrons that escaped the respiratory chain producing $O_2^-\cdot$ may re-reduce cytochrome *c* and still contribute to energy production by providing the energy needed to pump H$^+$ through Complex IV. Finally, the spontaneous dismutation of $O_2^-\cdot$ in the intermembrane space is facilitated by the lower pH in this compartment, resulting from the extrusion of H$^+$ coupled to respiration (Guidot *et al.* 1995).

Hydrogen peroxide, the product of $O_2^-\cdot$ dismutation and the main precursor of OH• in the presence of reduced transition metals, is mostly decomposed by the enzyme glutathione peroxidase. In the liver, mitochondria account for about one third of the total glutathione peroxidase activity (Chance *et al.* 1979). A second glutathione peroxidase associated with the mitochondrial membrane, known as phospholipid–hydroperoxide glutathione peroxidase, is specifically involved in reducing lipid peroxides associated with the membrane(Ursini *et al.* 1999; Nomura *et al.* 2000).

Catalase, a major $H_2O_2$ detoxifying enzyme found in peroxisomes, is also present in heart mitochondria (Radi *et al.* 1991). However, this enzyme has not been found in mitochondria from other tissues, including skeletal muscle (Phung *et al.* 1994).

In addition to cytochrome *c,* other electron carriers appear to have a detoxifying role against ROS. Ubiquinol (QH$_2$) has been shown to act as a reducing agent in the elimination of various peroxides in the presence of succinate (Bindoli *et al.* 1982; Eto *et al.* 1992). Thus, coenzyme Q is a source of $O_2^-\cdot$ when partially reduced (semiquinone form) and an antioxidant when fully reduced (Beyer, 1990). The inner mitochondrial membrane also contains vitamin E, a powerful antioxidant that interferes with the propagation of free radical-mediated chain reactions (Ham & Liebler, 1995).

Finally, cytochrome *c* oxidase (Complex IV) may also act as a peroxidase although, given the high $K_m$ for $H_2O_2$ (0.18 mM), the relevance of this reaction may be negligible (Orii, 1982).

In addition to the antioxidant defences mentioned above, the mitochondrion has a variety of DNA-repairing enzymes to correct errors resulting from oxidative damage. This is very important because, although 95 % of the mitochondrial proteins are encoded by the nuclear DNA, the mitochondrial chromosome (mtDNA) contains genes for several important proteins including subunits of NADH dehydrogenase and cytochrome oxidase and cytochrome *b*.

Although under normal conditions there is a balance between ROS formation and antioxidants, in several pathological scenarios the antioxidant defences become insufficient resulting in oxidative stress leading often to apoptosis and cell death. Apoptosis (or programmed cell death) is the mechanism used by mammals, plants and other organisms to eliminate redundant or damaged cells (Kroemer *et al.* 1998; Hoeberichts & Woltering, 2003). Apoptosis may be triggered by extracellular signals (extrinsic pathway) or by intracellular processes (intrinsic pathway). An increased mitochondrial formation of ROS triggers the intrinsic pathway by increasing the permeability of the outer mitochondrial membrane through the opening of transition pores. The opening of the permeability transition pore is favoured by oxidative stress through oxidation of intracellular glutathione and other critical sulfhydryl groups (Chernyak, 1997). As a result of this process, cytochrome *c* moves from the intermembrane space into the cell's cytoplasm (Liu *et al.* 1996) where it joins another factor (Apaf-1). In the presence of dATP this complex polymerizes into an oligomer known as 'apoptosome'. The apoptosome activates a protease (caspase-9), which in turn activates caspase-3. The cascade of proteolytic reactions also activates DNAses and in the end the process results in cell death (Li *et al.* 1997). Under normal conditions, various anti-apoptotic factors (including Bcl-xL) prevent the mitochondrial permeability transition as long as they remain bound to the outer membrane. This factor is eliminated when another factor, Bax, is translocated to mitochondria, starting apoptosis (Finucane *et al.* 1999).

The gradual loss of cytochrome *c* from the intermembrane space during apoptosis favours the mitochondrial formation of $O_2^-\cdot$ in two ways: (1) cytochrome *c* is a scavenger of $O_2^-\cdot$ and (2) as cytochrome *c* is released, the respiratory chain becomes more reduced because electron flow between Complex III and Complex IV slows down (Cai & Jones, 1998).

### Mitochondrial ROS formation during hyperoxia and during hypoxia

As predicted from eqn (1), the mitochondrial production of $O_2^-\cdot$ must increase with oxygen concentration. The proportion of oxygen converted into $O_2^-\cdot$ *in vitro* ($[O_2] = 220$ $\mu$M) accounts for about 1–2 % of the overall oxygen consumption. *In vivo*, particularly in tissues not exposed to atmospheric oxygen, the proportion of oxygen converted $O_2^-\cdot$ is likely to be smaller since the

intramitochondrial oxygen concentration is between 3 and 30 $\mu$M (Wittenberg & Wittenberg, 1989; Genova *et al.* 2001; Alvarez *et al.* 2003).

As oxygen concentration increases, the rate of mitochondrial $O_2^-\bullet$ production increases linearly (Turrens *et al.* 1982). However, the release of $H_2O_2$ from mitochondria is biphasic, increasing at a faster rate above 60 % $O_2$ (Turrens *et al.* 1985). The slower release of $H_2O_2$ at lower $P_{O_2}$ suggests that the mitochondrial antioxidant defences can compensate for sudden increases in the concentration of this peroxide. Apparently these defences become overwhelmed at higher $P_{O_2}$, which explains the mitochondrial alterations observed in the lungs of animals exposed to oxygen concentrations around 60 % or higher (Crapo *et al.* 1983).

Under normobaric hyperoxic conditions, the only organs affected by ROS formation are the lungs, since they the only ones in direct contact with atmospheric oxygen. However, under hyperbaric conditions, more oxygen is dissolved in the plasma, and therefore other tissues become exposed to a hyperoxic environment. Under these conditions, the brain is the first organ to show the effects of an increased ROS formation, resulting in convulsions. Interestingly, this phenomenon was proposed to be associated with oxygen free radicals 15 years before the discovery of $O_2^-\bullet$ as a normal intracellular metabolite (Gerschman *et al.* 1954).

The formation of ROS should decrease with hypoxia, since this activity is proportional to ROS (eqn (1)). Yet, various groups have reported a paradoxical increase in oxidative stress under moderately hypoxic conditions (1.5 % $O_2$, equivalent to an oxygen concentration of around 16 $\mu$M; Waypa & Schumacker, 2002; Schumacker, 2002). These studies show that when cells are incubated with dichlorofluorescein, a fluorescent probe for ROS, hypoxia increases fluorescence in cells with functioning mitochondria (Chandel *et al.* 2001). Mutants without a functioning respiratory chain do not show this increase in fluorescence (Chandel *et al.* 2001; Schroedl *et al.* 2003). This response is eliminated when cells are made severely hypoxic (Schumacker, 2002).

It has been proposed that the increase in ROS formation during hypoxia may modulate the activation of one or more hypoxia-inducible factors (HIF; Chandel *et al.* 2001), a group of proteins that regulate the expression of genes involved in the adaptation to hypoxic conditions (Semenza, 2002). In the presence of oxygen, proline residues 402 and 564 of HIF-1 $\alpha$ become hydroxylated, which eventually leads to ubiquitination and degradation of this factor (Semenza, 2002), thus preventing the induction of a hypoxic stress response. Schroedl *et al.* suggested that the stabilization of HIF under hypoxic conditions requires mitochondrial ROS formation, as this

process is lost in cells without a functioning respiratory chain (Schroedl *et al.* 2003). They proposed that the mitochondrial formation of ROS is required to stabilize HIF-1 $\alpha$ under hypoxic conditions while the proline hydroxylase activity would be involved in an 'on or off' type of response under anoxic conditions (Schroedl *et al.* 2003).

The proposed increase in ROS formation during hypoxia is difficult to explain. Given the high affinity of cytochrome oxidase for oxygen, at low $P_{O_2}$ any remaining oxygen should be reduced to water by the terminal oxidase. Two factors may contribute to an increase mitochondrial $O_2^-\bullet$ formation. Firstly, under hypoxic conditions, low concentrations of NO$\bullet$ may still be produced (5–10 % of the normal steady state) since the $K_m$ for oxygen of the mitochondrial nitric oxide synthase is around 30–40 $\mu$M (Alvarez *et al.* 2003). Secondly, nitric oxide may bind and inhibit cytochrome oxidase, resulting in an increase in its $K_m$ for oxygen and an increased reduction of electron carriers located upstream from the terminal oxidase (Cooper & Davies, 2000), favouring $O_2^-\bullet$ formation at low oxygen concentrations. Still more research is needed to clarify the extent of ROS formation in the response of tissues to hypoxia.

## Mitochondrial ROS formation resulting from exposure to xenobiotics

Several xenobiotics interact with the mitochondrial electron transport chain, increasing the rate of $O_2^-\bullet$ production through two different mechanisms. Some of these compounds stimulate oxidative stress because they block electron transport, increasing the reduction level of carriers located upstream of the inhibition site. Other xenobiotics may accept an electron from a respiratory carrier and transfer it to molecular oxygen (redox cycling), stimulating $O_2^-\bullet$ formation without inhibiting the respiratory chain.

It has been proposed that Parkinson's disease may result from exposure to sublethal concentrations of inhibitors of Complex I. The current hypothesis is that these inhibitors stimulate $O_2^-\bullet$ formation by Complex I, causing the cells to undergo apoptosis (Sherer *et al.* 2003*a*,*b*). Compounds such as MPP$^+$ (1-methyl-4-pyridinium), a metabolite of MPTP (*N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a toxic 'designer drug' from the 1980s which causes a Parkinson's-like syndrome, block electron flow through Complex I (Trojanowski, 2003). Moreover, exposure of animals to rotenone is accompanied by formation of deposits of $\alpha$-synuclein similar to those observed in various related brain amyloidoses, suggesting that inhibition of Complex I may have an important role in other neuropathies (Trojanowski, 2003).

The antitumour agent adriamycin (doxorubicin) and other anthracyclines constitute examples of redox cycling

xenobiotics. These compounds accept electrons directly from Complex I, causing myocardial toxicity through an increase in the steady-state concentration of $O_2^-\bullet$ (Jung & Reszka, 2001).

## Changes in mitochondrial antioxidant defences: apoptosis and ageing

Oxidative stress may also result from deficiencies in antioxidant defences. Both genetic factors and ageing may cause an increased mitochondrial steady-state concentration of ROS.

The first example involves the mitochondrial form of SOD. The gene for MnSOD (also known as SOD2) is located in the nuclear DNA and the enzyme must be transported into the matrix after translation, where it forms the active homotetramer (Fridovich, 1995; Sutton *et al.* 2003). The efficiency of MnSOD transport depends upon the mitochondrial targeting sequence (MTS), a signal polypeptide that causes the protein to be imported into the matrix. The sequence for this MTS presents a genetic dimorphism, in which the amino acid alanine in the ninth position is replaced by valine. In this case the tertiary structure of the MTS is altered and the change in tertiary structure interferes with MnSOD translocation into the matrix, resulting in a 40 % lower SOD activity in this compartment (Sutton *et al.* 2003). At least two reports have correlated this mutation with an increased incidence of Parkinson's disease, stressing the correlation between oxidative stress and this disease (Shimoda-Matsubayashi *et al.* 1996; Grasbon-Frodl *et al.* 1999).

Amyotrophic lateral sclerosis (ALS) is another neurological disease that has been associated with oxidative stress. About 10 % of the cases of familial ALS have been linked to a mutation in the gene coding for CuZnSOD (Valentine & Hart, 2003). This mutation causes misfolding, and also prevents the effective import of this enzyme to the inter-membrane space, thus increasing the steady-state concentration of $O_2^-\bullet$ in this compartment, a process that may lead to apoptosis (Okado-Matsumoto & Fridovich, 2002).

Another genetic mutation indirectly associated with increased formation of ROS is the mutation in one of the subunits of Complex I responsible for Leber hereditary optic neuropathy that causes neuronal apoptosis. In a recent article, the investigators manipulated the expression of MnSOD in order to increase the intramitochondrial steady state of $O_2^-\bullet$ in normal cells, resulting in the same histopathological changes observed in Leber's disease (Qi *et al.* 2003).

Although mitochondria express a variety of protective defences (antioxidant and repair enzymes as well as low molecular weight antioxidants), it has been proposed that the oxidation of proteins and the slow accumulation of

DNA lesions resulting from the continuous formation of ROS may contribute to the ageing process (Sohal *et al.* 1993; Ames *et al.* 1995). Some of these lesions affect the rate of electron flow and lead to an increased formation of ROS, which supports the observed correlation between the rate of mitochondrial $O_2^-\bullet$ and $H_2O_2$ formation and lifespan among several species (Lopez-Torres *et al.* 1993; Ku *et al.* 1993).

The ageing process has also been associated with deficiencies in the mitochondrial DNA repair system. The mitochondrial DNA does not contain histones, and therefore is less protected against oxidative stress than the nuclear DNA. As a result the mitochondrial DNA shows a 10- to 20-fold increase in the content of 8-hydroxyguanine, the product of guanine oxidation (McCord & Fridovich, 1988). Since the mitochondrial chromosome codes for some electron carriers, mtDNA damage may indirectly inhibit respiration and stimulate ROS formation. Cockayne syndrome, a human condition that causes premature ageing, has been associated with a deficiency in the mitochondrial enzyme required for DNA repair that catalyses the removal of 8-hydroxyguanine (Bohr *et al.* 1998).

Other studies have shown a more direct correlation between oxidative stress and ageing. First, there is a correlation between accumulation of oxidized proteins and lifespan, further linking oxidative stress with ageing (Sohal *et al.* 1993). In a more direct approach, another study showed that overexpression of catalase and SOD results in a 25 % increase in the lifespan of *Drosophila melanogaster* (Orr & Sohal, 1994).

The sex-related differences in lifespan across species, with females usually living longer than males, also correlate with differences in antioxidant defences. This difference appears to be associated with the production of oestrogens, since it is not observed in ovariectomized animals (Asdell *et al.* 1967). Although the mechanism behind this effect has not yet been elucidated, part of the oestrogen-dependent protection is associated with an increased mitochondrial concentration of glutathione and glutathione peroxidase activity (Borras *et al.* 2003).

## Conclusion

The mitochondrial respiratory chain constitutes the main intracellular source of ROS in most tissues. The steady-state concentration of these oxidants is maintained at non-toxic levels by a variety of antioxidant defences and repair enzymes. The delicate balance between antioxidant defences and ROS production may be disrupted by either deficient antioxidant defences, inhibition of electron flow or exposure to xenobiotics. This imbalance appears as a common denominator in various pathological processes in which the resulting oxidative insult causes tissue damage and, eventually, cell death.

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