



Production of Free Radicals by Mitochondria: A Biochemistry Approach

Usman Garba Kurmi¹⁾, Fatima Abubakar²⁾, Ruqayya Ali Uthman³⁾,
Isah Adamu Baushe⁵⁾, Hadiza Abubakar Balkore⁷⁾, Khadija Muhammad⁴⁾,
Prosper Obed Chukwuemeka⁶⁾

^{1), 7)}University of Maiduguri, Faculty of Science, Department of Biochemistry, Nigeria

²⁾University of Maiduguri, Faculty of Science, Department of Microbiology, Nigeria

³⁾Bayero University Kano, Faculty of Basic Health Science, Department of Biochemistry, Nigeria

⁴⁾Kaduna State University, Faculty of Science, Department of Biochemistry, Nigeria

⁵⁾Yobe State University, Faculty of Science, Department of Chemistry, Nigeria

⁶⁾Federal University of Technology Akure, School of Sciences (SOS), Department of Biotechnology, Nigeria

Email: usmankurmi@yahoo.com¹⁾, teeemadk@gmail.com²⁾, abubakarbalkoreh@gmail.com⁷⁾,
ruqayyaalee@gmail.com³⁾, hadizamuhammad188@gmail.com⁴⁾, isahadamubaus@gmail.com⁵⁾,
chukwuemekamcb154271@futa.edu.ng⁶⁾

Abstract

The production of superoxide by mitochondria strongly depends on the mitochondrial respiratory chain. It is believed that the acetylation of cytochrome c prevents its reoxidation by mitochondrial cytochrome oxidase and direct reduction by NADH-or succinate-dependent cytochrome c reductase. It is believed that ubiquinone can be one of the major producers of superoxide because the depletion of endogenous ubiquinone resulted in the formation of diminishing hydrogen peroxide.

Keywords: biotechnology, biochemistry, mitochondria

1. Background

Although the first manifestations of hydrogen peroxide formation by mitochondria [1,2] have been taken with caution, the following studies [3,4] confirmed these findings. It has been reasonable to suggest that hydrogen peroxide could be a product of superoxide dismutation and that superoxide is produced by mitochondria. Such proposal was indeed confirmed by Loschen et al. [5] and Boveris and Cadenas [6], who showed that hydrogen peroxide is a stoichiometric precursor of mitochondrial hydrogen peroxide. The production of superoxide by mitochondria strongly depends on the mitochondrial respiratory chain. As is well known, respiratory chain consists of a series of electron carriers (pyridine nucleotide, flavoproteins, iron-sulfur proteins, ubiquinone, and cytochromes), which are arranged on the mitochondrial inner membrane according to their redox potentials from 320 to p380mV. It is usually accepted that up to 97% of dioxygen consumption by mitochondria is converted by cytochrome oxidase into water and only 1–3% is leaked to be reduced into superoxide production.

2. Discussion

2.1 Detection of Active Oxygen Species in Mitochondria

Controversial results of oxygen radical detection in mitochondria have been described in the literature. Owing to experimental difficulties many authors were obliged to work with submitochondrial particles instead of the whole mitochondria. However, it is quite possible that oxygen radical production by submitochondrial particles may be artificially enhanced due to exposure to oxygen. On the other hand, some analytical methods of superoxide detection such



as cytochrome c reduction cannot be used due to the direct reduction of cytochrome by mitochondrial components. In earlier studies [5,6] superoxide detection in mitochondria was equated to hydrogen peroxide formation. However, while it is quite possible that superoxide is a stoichiometric precursor of mitochondrial hydrogen peroxide, it is understandable that the level of hydrogen peroxide may be decreased due to the reactions with various mitochondrial oxidants. Moreover, superoxide level can be underestimated due to the reaction with mitochondrial MnSOD. Several authors [7,8] assumed that mitochondrial superoxide production may be estimated through cyanide-resistant respiration, which supposedly characterizes univalent dioxygen reduction. This method was applied for the measurement of superoxide production under in vitro normoxic and hyperoxic conditions, in spite of the finding [7] that cyanide-resistant respiration reflects also the oxidation of various substrates (lipids, amino acids, and nucleotides). Earlier, it has been suggested that superoxide can be measured in mitochondria by the SOD-inhibitable oxidation of adrenaline to adrenochrome and the reduction of acetylated cytochrome c [8–10]. It is believed that the acetylation of cytochrome c prevents its reoxidation by mitochondrial cytochrome oxidase and direct reduction by NADH- or succinate-dependent cytochrome c reductase. A very interesting method has been proposed by Ksenzenko et al. [11], who used ESR spectroscopy for the measurement of superoxide production in mitochondria on the basis of Tiron (1,2-dihydroxybenzene-3,5-sulfonate) semiquinone formed in the reaction with superoxide.

2.2 Rates and Mechanism of Superoxide Production in Mitochondria

Despite a long-time studying of superoxide production by mitochondria, an important question is still debated: does mitochondria produce superoxide under physiological conditions or superoxide release is always a characteristic of some pathophysiological disorders resulting in the damage of normal mitochondrial functions? Uncertainties in this question arise due to the different results obtained with the use of respiratory inhibitors and different analytical methods. Usually, mitochondrial superoxide production is registered only after the incubation of submitochondrial particles with respiratory inhibitors, first of all, rotenone and antimycin. Under such conditions, superoxide production may achieve about $1 \text{ nmol} / 1 \text{ min} / 1 \text{ mg}$ of protein [14]. The rates of superoxide production depend on both tissue oxygen tension and the redox state of respiratory chain carriers. For example, hyperoxia increases superoxide production in rat lung mitochondria [7] and lung submitochondrial particles [8]. It has also been shown that hydrogen peroxide production by mitochondria depends on the mitochondrial metabolic state, and is higher in State 4, which is characterized by a slow respiratory rate and a high reduction level of electron carriers, and lower in State 3 where electron carriers are largely oxidized [15]. As superoxide is a precursor of hydrogen peroxide, the same may be said about the formation rates of hydrogen peroxide. The most convincing proof of mitochondrial superoxide production under physiological conditions was presented by Li et al. [12] who has found that the mitochondria isolated from monocyte or macrophages generated significant lucigenin-amplified CL in the presence of substrates and without any respiratory inhibitors. It should be stressed that the efficiency of superoxide detection depends on the capacity of superoxide scavengers to achieve the sites of superoxide production in mitochondria. It has been shown [16,17] that the superoxide release takes place in the mitochondrial matrix or the intermembrane space. Therefore, lucigenin, which is able to enter the mitochondrial matrix, can quantitatively register superoxide. However, it should be noted again that superoxide concentration under physiological condition might be underestimated by mitochondrial MnSOD [18].



Now, we may consider in detail the mechanism of oxygen radical production by mitochondria. There are definite thermodynamic conditions, which regulate one-electron transfer from the electron carriers of mitochondrial respiratory chain to dioxygen: these components must have the one-electron reduction potentials more negative than that of dioxygen. Furthermore, there are probably at least two sites of mitochondrial superoxide production: the NADH ubiquinone reductase (Complex I) and the ubiquinol–cytochrome c reductase (Complex III). It is believed that ubiquinone can be one of the major producers of superoxide because the depletion of endogenous ubiquinone resulted in the formation of diminishing hydrogen peroxide [21]. Furthermore, Complexes I and III, which contain ubiquinone as a major component, are the effective producers of superoxide and hydrogen peroxide [20].

As the reduction potentials of components of respiratory chain are changed from 0.320 to 0.380 V, it is obvious that various sources of superoxide production may exist in mitochondria. As already noted earlier, the two main sources of superoxide are present in Complexes I and III of the respiratory chain; in both of them, the role of ubiquinone seems to be dominant. Although superoxide may be formed by the one-electron oxidation of ubiquinone radical anion (Reaction (1)) [10,22] or even neutral semiquinone radical [9], the efficiency of these ways of superoxide formation in mitochondria is doubtful.

2.3 Free Radical-Mediated Damage to Mitochondria

Free radical-mediated damage to mitochondria originates from two sources: active oxygen species (superoxide, hydrogen peroxide, and secondary radicals) formed in mitochondria itself and free radicals, which attack mitochondria from outside. Early studies demonstrated that active oxygen species produced by xanthine oxidase impaired the ability of mitochondria to take and retain calcium [29], decreased the State 3 respiration [30], and reduced the pyruvate-induced mitochondrial respiration [31]. Other exogenous sources of free radical-mediated damage are xenobiotics and drugs, which can be reduced by mitochondria. Davies and Doroshow [33,34] demonstrated that the anthracycline antibiotics doxorubicin and daunorubicin are reduced to semiquinones by Complex I of cardiac mitochondria and enhance the production of superoxide, hydrogen peroxide, and probably hydroxyl radicals. Menadione also stimulated superoxide production and calcium release from rat liver mitochondria [35]. Kohda and Gemba [36] suggested that the cephaloridine-induced nephrotoxicity in rats might be a consequence of the enhanced superoxide production in kidney cortical mitochondria. Another stimulator of superoxide production in mitochondria is ethinyl estradiol, a strong promoter of hepatocarcinogenesis. It was demonstrated [37] that ethinyl estradiol increased superoxide production by rat liver mitochondria under in vitro and in vivo conditions. Calcium oxalate monohydrate responsible for the formation of most kidney stones significantly increased mitochondrial superoxide production in renal epithelial cells [42]. Recombinant human interleukin IL-1 β induced oxygen radical generation in alveolar epithelial cells, which was suppressed by mitochondrial inhibitors 4'-hydroxy-3'-methoxyacetophenone and diphenylene iodonium [43]. Esposito et al. [44] found that mitochondrial oxygen radical formation depended on the expression of adenine nucleotide translocator Ant1. Correspondingly, mitochondria from skeletal muscle, heart, and brain from the Ant1-deficient mice sharply increased the production of hydrogen peroxide.

2.4 Mechanisms of Mitochondria Protection from Free Radical-Mediated Damage

There are different pathways for the regulation and suppression of free radical production in mitochondria. Thus, Skulachev [54,55] proposed that the overproduction of free radicals in



mitochondria may be suppressed by molecular processes such as the maintenance of low dioxygen levels, strong uncoupling, and the release of mitochondrial suicide proteins Skulachevase, and cytochrome c. Endogenous antioxidants (ubiquinol, glutathione, and vitamin E) and the antioxidant enzymes (first of all, MnSOD) play an important role in preventing free radical-mediated damage. For example, Lass and Sohal [56] have shown that the oral administration of α -tocopherol to mice suppressed superoxide production by submitochondrial particles; the same results were obtained under in vitro conditions. In contrast, the administration of ubiquinol Q10 had no effect on mitochondrial superoxide production. Furthermore, defensive mechanisms in mitochondria against free radical damage depend on the relative activities of antioxidant enzymes. Mitochondrial glutathione peroxidase apparently plays a key role in destroying hydrogen peroxide due to the absence of catalase in mitochondria of most of the animal cells [57]. For regeneration of reduced glutathione (an important mitochondrial antioxidant) and maintaining the activities of glutathione reductase and peroxidase systems mitochondria requires NADPH. Jo et al. [58] have shown that NADPb-dependent isocitrate dehydrogenase (ICDH) is a major NADPH producer in mitochondria and therefore, an important factor in fighting against free radical-induced damage. Correspondingly, the decreased expression of ICDH significantly enhanced free radical production, DNA fragmentation, and lipid peroxidation in mitochondria. It has been found [59] that nitric oxide inactivated ICDH supposedly through S-nitrosylation of its cysteine residues.

At present, the study of apoptosis, physiological cell death, is probably the most rapidly growing part of biological studies relevant to free radical research. Apoptosis is a two-stage process, in which mitochondria plays an important role [82]. The first stage is characterized by the attack of mitochondria by various physiological and pathophysiological stimuli, which increases mitochondrial membrane permeability. Mitochondrial permeability leads to the formation of a dynamic multiprotein complex at the site between the inner and outer mitochondrial membranes. Inhibition of the formation of this complex (for example, by mitochondrial expression of oncoprotein Bcl-2) prevents the development of apoptosis. At the second stage, mitochondrial dysfunction is accompanied by uncoupling of the respiratory chain and overproduction of free radicals.

2.5 Reactive Oxygen Species as Mediators of Apoptosis

Free radicals have been implicated as important modulators of apoptosis in many studies, although the experiments carried out under hypoxic conditions suggested that apoptosis might occur in their absence. There are numerous apoptotic pathways induced by different stimuli, and reactive oxygen species may affect them in different ways. It has been proposed that gene-induced apoptosis is characterized by the transcriptional induction of redox-related genes, the formation of reactive oxygen species, and the oxidative degradation of mitochondrial components, culminating in cell death [83]. Johnson et al. [84] have showed that regulated the level of reactive oxygen species in vascular smooth muscle cells (VSMCs), which are generated concomitantly with an increase in development and the onset of apoptosis. The functional role of oxygen species in the development of stimulated apoptosis was supported by the inhibitory effects of antioxidants (pyrrolidine dithiocarbamate (PDTC), N-acetylcysteine, and catalase) on both oxygen radical generation and apoptosis. Cai and Jones [85] also found that the overproduction of oxygen radicals occurred in staurosporine-treated HL60 cells, and that the release of cytochrome c, a key event in the development of apoptosis, was associated with inhibited mitochondrial respiration and stimulated superoxide production. However, they pointed out that another key factor of apoptosis, the activation of "death protease" caspase 3



was independent of oxygen radical formation.

Nonetheless, the stimulation of apoptosis by reactive oxygen species has been shown in many other studies with the use of different apoptotic stimuli. For example, it has been suggested that oxygen species mediate the transforming growth factor beta (TGF- β)-induced apoptosis in fetal hepatocytes [86,87]. In this case TGF- β -induced apoptosis was suppressed by free radical scavengers (ascorbate and PDTC). A decrease in superoxide production inhibited T cell apoptosis [88]. Hsieh et al. [89] have shown that oxidized LDL induced apoptosis in VSMC, which was mediated by reactive oxygen species. As the formation of oxygen species was inhibited by rotenone and nordihydroguaiaretic acid (the lipoxygenase inhibitor), mitochondrial and lipoxygenase pathways can be involved. Blatt et al. [90] have studied the effect of proapoptotic 1,4-benzodiazepine Bz-423 on the transformed Ramon B cells. It was found that Bz-423 induced superoxide formation, which functioned as an upstream signal that initiated cytochrome c release, mitochondrial depolarization, and caspase activation. Priault et al. [91] demonstrated that the expression of proapoptotic protein Bax induced the oxidation of mitochondrial lipids. It was concluded that Bax-induced lipid peroxidation, and not superoxide or hydrogen peroxide formation is relevant to Bax-stimulated apoptosis.

The ras protooncogene is a well-known modulator of apoptosis. For example, it has been demonstrated [92] that apoptosis occurred in the protein kinase C (PKC)-inhibited rastransformed NIH/3T3 cells. The treatment with N-acetylcysteine and culturing the cells in low oxygen conditions suppressed the apoptotic response to PKC inhibition. It was concluded that oxygen species apparently mediated the ras-induced apoptosis, but they are not only obligatory mediators because continued cell progression is also necessary for the induction of apoptosis under these conditions. Another effective apoptotic stimulus is *Vibrio vulnificus* cytotoxin (VVC), a water-soluble polypeptide isolated from a marine bacteria, [95] which causes wound infections and septicemia [93]. It was proposed that VVC-stimulated apoptosis in human vascular endothelial cells is triggered by superoxide elevation, which is accompanied by the release of cytochrome c, the activation of caspase 3, the cleavage of poly(ADP-ribose) polymerase, and DNA fragmentation. Zinc induced apoptosis in mammary cancer cells, which was apparently mediated by the formation of oxygen species and the Fas/Fas induction [94].

2.6 Mechanisms of the Activation of Apoptosis by Reactive Oxygen Species

After considering relevant to the participation of mitochondrial reactive oxygen species in the development of apoptosis, we may now regard mechanisms of the oxygen species activation of apoptosis [77]. Although major events and main participants of oxygen radical-mediated apoptotic pathways are well known, there is no full agreement in their roles and the succession of the events. As superoxide is a well-proven participant in apoptosis, and its role is tightly connected with the release of cytochrome c. It has been proposed that a switch from the normal four-electron reduction of dioxygen through mitochondrial respiratory chain to the one-electron reduction of dioxygen to superoxide can be an initial event in apoptosis development [96].

Superoxide may activate other apoptotic pathways with or without stimulating the cytochrome c release. It is of interest that superoxide and hydrogen peroxide might induce apoptosis by different ways [11]. Thus, von Harsdorf et al. [57] demonstrated that superoxide-induced apoptosis in cardiomyocytes was not accompanied by cytochrome c release and was associated with an increase in apoptotic protein level. In studying drug-induced apoptosis in tumor cells Hirpara et al. [89] showed that such drugs stimulated superoxide production which was



followed by cytochrome c release. They proposed that apoptosis depended on acidification triggered by hydrogen peroxide and cytochrome c release, with both factors acting as signals for the activation of caspase cascade.

2.7 Protection Against Apoptosis Activated by Reactive Oxygen Species

The biggest difficulty in understanding the role of reactive oxygen species in apoptosis are the findings showing that the same oxygen radicals, which activate apoptosis may, under certain conditions, inhibit it. Correspondingly, antioxidants, which predictably must inhibit oxygen radical-stimulated apoptosis, are also able to activate apoptosis under certain conditions. For example, superoxide, an effective promoter of apoptosis, at the same time may suppress Fas-mediated apoptosis [84]. It has been suggested that reactive oxygen species are able to inhibit caspases [15], exhibiting antiapoptotic instead of proapoptotic effects. Furthermore, superoxide generation may activate the transcription factor NF- κ B, which suppresses TNF- α -induced apoptosis [96]. Skulachev [17] proposed that the released cytochrome c oxidizes superoxide and, by this, exhibits an antioxidant function. This proposal was supported by recent experimental findings by Atlante et al. [88], who suggested that cytochrome c released from mitochondria by oxygen species protected mitochondria through a feedback-like process oxidizing superoxide. The most important physiological inhibitor of apoptosis is multifunctional protein Bcl-2, which is localized on the mitochondrial outer membrane.

2.8 Mitochondrial Nitrogen Oxide Production and Apoptosis

Similar to reactive oxygen species, nitric oxide, peroxynitrite, and other nitrogen oxide species produced by mitochondria are able to stimulate or inhibit apoptosis. Proapoptotic effect of nitric oxide was probably first shown by Albina et al. [98], who demonstrated NO-induced apoptosis in macrophages. Since then, NO-stimulated apoptosis was found in many primary cell types such as macrophages, pancreatic cells, thymocytes, and neurons [20]. NO-stimulated apoptosis in vascular smooth muscle cells apparently involves the activation of soluble guanylyl cyclase [97]. It has been proposed that apoptosis by nitric oxide is a consequence of DNA damage, which leads to the accumulation of proapoptotic tumor suppressor protein [91]. Cheng et al. [142] found that nitric oxide induced apoptosis in neural progenitor cells, which was mediated by the activation of MAP kinase, poly (ADP-ribose) polymerase, and caspase 3. Antiapoptotic protein Bcl-2 protected progenitor cells against NO-induced apoptosis and inhibited the activation of MAP kinase. Nitric oxide also stimulated apoptosis in human and rat pulmonary artery smooth muscle cells by activating K(Ca) and K(v) channels in the plasma membrane [98].

3. Conclusion

In conclusion, it was suggested that the activation of protein kinases (for example, protein kinase C or tyrosine kinase) may stimulate the phosphorylation of KATP channels, causing channel opening. However, concluded that it is the KATP channel opening that increases the production of oxygen species, which triggers the entrance into a PC state. It is possible that the disagreement in the effects of hypoxia on oxygen radical production obtained by different authors could be related to the differences in preparations and analytical methods.

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