AUCTORES
Globalize your Research

Biomedical Research and Clinical Reviews

Bon L.I *

Open Access

Short Communication

Generation of Free Radicals and Antioxidant Protection of Mitochondria

Fliuryk S., Dremza I., Bon L.I *., Malenovskaya M.Y., Senko N.S., Rud I.G., Tomchuk A.M., Malafei A.A

Candidate of Biological Science, Associative Professor Grodno State Medical University, Belarus

***Corresponding Author:** Bon L.I, Candidate of Biological Science, Associative Professor Grodno State Medical University, Belarus.

Received Date: August 03, 2026; Accepted Date: August 10, 2026; Published Date: August 20, 2026

Citation: Fliuryk S., Dremza I., Bon L.I., Malenovskaya M.Y., Senko N.S., et al, (2026), Generation of Free Radicals and Antioxidant Protection of Mitochondria, *J. Biomedical Research and Clinical Reviews*, 12(5); **DOI:10.31579/2690-4861/267**

Abstract

The main source of reactive oxygen species (ROS) in the cell is mitochondrial respiration. It was first shown that isolated mitochondria from the liver and heart of animals are active sources of hydrogen peroxide (H_2O_2), which is capable of diffusing into the cytosol.

Key words: reactive oxygen species; ubisemiquinone; mitochondria

Introduction

The main source of reactive oxygen species (ROS) in the cell is mitochondrial respiration. It was first shown that isolated mitochondria from the liver and heart of animals are active sources of hydrogen peroxide (H_2O_2), which is capable of diffusing into the cytosol [1,2]. It is assumed that in the heart and liver of rats, mitochondrial H_2O_2 production accounts for approximately 0.5% of the total organ O_2 consumption when mitochondria use substrates such as succinate or malate-glutamate, and about 0.15% when palmitoyl-carnitine is used as a substrate. Somewhat later, it was established that the precursor of mitochondrial H_2O_2 is superoxide ($O_2^{\cdot-}$), and its main source in mitochondria was identified as the autoxidation of ubisemiquinone (UQH·) [2,3]. It is currently known that $O_2^{\cdot-}$ is formed not only during the autoxidation of UQH·, but also due to electron leakage from complexes I, II, and III of the electron transport chain (ETC) to molecular oxygen.

Significant progress in the study of mitochondrial free radical processes was the demonstration of the kinetics of $O_2^{\cdot-}$ formation in the mitochondria of living cells and organs. Thus, in cell cultures loaded with a new $O_2^{\cdot-}$ biosensor (yellow fluorescent protein), researchers observed fluorescence bursts in individual mitochondria or groups of functionally connected mitochondria, reflecting $O_2^{\cdot-}$ production [4]. The distinctive feature of this fluorescent protein was that it did not react with other ROS in the cell, such as H_2O_2, peroxynitrite, hydroxyl radical, or NO [4]. The result of $O_2^{\cdot-}$ release was the opening of mitochondrial permeability transition pores (mPTP) not associated with Ca^{2+} release. $O_2^{\cdot-}$-initiated mPTP opening was associated with ETC activity and was ATP-dependent. The generation of $O_2^{\cdot-}$ correlated with a transient decrease in membrane potential, matrix acidification, and mitochondrial swelling. Anoxia or moderate hypoxia reduced the frequency of $O_2^{\cdot-}$ fluorescence flashes in cardiomyocytes, highlighting the role of selective ROS production in the cell. This process utilizes scavenging and detoxifying systems in signaling cascades, as well as kinases, phosphatases, membrane channels, and transporters. However, some researchers [5] have disputed the idea that mitochondria are the main producers of $O_2^{\cdot-}$ and H_2O_2 in mammalian cells, arguing that the generation of free radicals by mitochondria may be an artifact resulting from disruption of mitochondrial membrane integrity during isolation [6]. These same authors showed that disruption of redox coupling at the ubiquinone/cytochrome bc1 site, which depends on the physical state of the inner mitochondrial membrane, is accompanied by electron leakage and ROS generation.

The second source of ROS in the cell comprises certain mitochondrial enzymes, in particular two enzymes of the Krebs cycle — aconitase and α-ketoglutarate dehydrogenase — as well as pyruvate dehydrogenase, glycerol-3-phosphate dehydrogenase, dihydroorotate dehydrogenase, monoamine oxidases A and B, and cytochrome b5 reductase.

The third important source of mitochondrial ROS production is mitochondrial NO synthase (mtNOS) [7,8]. In hepatocytes and cardiomyocytes, NOS is localized inside the mitochondria, whereas in endothelial cells it is associated with the intermembrane side of the outer mitochondrial membrane [8].

The majority of $O_2^{\cdot-}$ (70–80%) formed in the mitochondrial ETC enters the matrix, while 20–30% enters the intermembrane space. Previously, two main pathways of $O_2^{\cdot-}$ production in the mitochondrial ETC were described in detail: 1) as a result of the autoxidation of intermediate semiquinones (UQH· for the redox pair ubiquinol/ubiquinone: $UQH\cdot + O_2 \rightarrow UQ + H^+ + O_2^{\cdot-}$) [2], and 2) as a result of the oxidation of the coenzyme FMNH2/FMN by NADH dehydrogenase ($FMNH\cdot + O_2 \rightarrow FMN + H^+ + O_2^{\cdot-}$) [9]. It is known that semiquinones are non-enzymatically oxidized by molecular O_2, forming $O_2^{\cdot-}$. It has been proven that ubisemiquinone is the main quantitative source of one-electron reduction of O2 in the mitochondrial ETC [10].

The formation of H_2O_2 in mitochondria is modulated by their metabolic state and the intramitochondrial concentration of NO. Thus, H_2O_2 production in metabolic state IV is approximately 4–5 times higher than in metabolic state III and amounts to 0.3–0.8 nmol $H_2O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$ in state IV and only 0.05–0.15 nmol $H2O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$ in state III [11]. Ion fluxes across the inner mitochondrial membrane have a strong effect on the rate of H_2O_2 formation, indicating a regulatory role of the membrane potential in the process of UQH· autoxidation [12].

The synthesis of nitric oxide catalyzed by mtNOS occurs in an enzymatic reaction that requires arginine, NADPH, and O_2 as substrates. As a result of the reaction, citrulline, H_2O, and NO are formed (NADPH + arginine + $O_2 \rightarrow NADP^+ + H_2O$ + citrulline + NO). Three different genomic NOS isoforms are known: neuronal NOS (nNOS or NOS_1), inducible NOS of macrophages (iNOS or NOS_2), and endothelial NOS (eNOS or NOS_3). Two independent groups of researchers have established that one of the sources of nitric oxide is the mitochondria [13,14]. Mitochondrial NO is produced by one of the isoforms of NOS — mitochondrial nitric oxide synthase. Physiological production of NO by the enzyme is interrelated with the regulation of cytochrome c oxidase activity: an increase in NO levels reduces cytochrome c oxidase activity through S-nitrosylation of cysteine residues in complexes IV and I [10,15]. In the uncoupled state, endothelial NOS (eNOS) generates the superoxide anion radical [16]. Uncoupling does not require dissociation and monomerization of the enzyme; it most likely occurs as a result of substrate and tetrahydrobiopterin deficiency, leading to facilitated superoxide generation through a NO-dependent mechanism and accompanied by peroxynitrite formation with a decrease in NO levels. Numerous pathological conditions lead to eNOS uncoupling; for example, this was clearly demonstrated in endothelial dysfunction [17].

Subsequently, mitochondrial NO synthase was identified as a variant of post-translational modification of nNOS involving its myristoylation and phosphorylation [18]. For the enzymatic reaction of NO synthesis to occur, the concentrations of NADPH, arginine, O_2, and Ca^{2+} in mitochondria must be sufficient. Mitochondrial and submitochondrial preparations synthesize NO at a rate of 0.25–0.90 $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$. It was calculated that the intramitochondrial steady-state concentrations of NO are 50–200 nM [10], and the NO release measured electrochemically in isolated mitochondria after the addition of Ca^{2+} amounted to 29 nM [20]. Under physiological conditions, tissue O_2 saturation is approximately 25 μM, and the mitochondrial $[O_2]/[NO]$ ratio is 150–300, which supports 25–50% inhibition of cytochrome oxidase [21]. It has been shown that NO produced by NO donors or mtNOS inhibits complex III of the ETC (50% inhibition of electron transfer between cytochromes b and c occurs at an NO concentration of 0.2–0.4 μM) and increases the production of $O2^{\cdot -}$ and H_2O_2 in mitochondria and submitochondrial particles [22]. This mechanism is often involved in the molecular mechanisms of neurological diseases. However, controversies regarding the existence of mtNOS still remain [19].

Intramitochondrial reactive oxygen and nitrogen species, such as $O_2^{\cdot -}$, H_2O_2, NO, and $ONOO^-$, are potential activators of oxidative stress, which is considered a factor determining the molecular mechanisms of tissue dysfunction in inflammation, neurological diseases, and aging. Two of them, $O_2^{\cdot -}$ and NO, are free radicals; however, due to their low reactivity, they do not participate in branching reactions but only in chain-terminating reactions, accompanied by the formation of H_2O_2 and $ONOO^-$. The latter two compounds are potentially dangerous because, after homolytic cleavage, they participate in the generation of the highly reactive hydroxyl radical (·OH) and the free radical $NO_2\cdot$, which is involved in protein nitration [23].

The formation of NO and H_2O_2 in rat liver mitochondria has an exponential dependence on the magnitude of the membrane potential [24]. In the mitochondrial matrix, the metabolism of NO and $O_2^{\cdot -}$ is not limited by diffusion in the reaction leading to the formation of peroxynitrite ($NO + O_2^{\cdot -} \rightarrow ONOO^-$) [25]. This oxidative pathway of NO utilization (80%) is the main one in metabolism and consumes only a small portion of $O_2^{\cdot -}$ (15%), whereas the reduction of NO by ubiquinol and cytochrome oxidase accounts for approximately 20% of NO utilization in mitochondria [10].

Peroxynitrite is a strong oxidant that, in its charged form, rapidly diffuses from the intramitochondrial space. Peroxynitrite irreversibly inhibits complexes I and III [26]; a significant increase in $ONOO^-$ levels in mitochondria leads to their dysfunction and cell apoptosis. The level of $ONOO^-$ in the mitochondrial matrix under physiological conditions is in the range of 2–5 nM [10], while levels above 20–30 nM are considered cytotoxic. Mitochondrial dysfunction is associated with excess NO and $ONOO^-$ during ischemia/reperfusion, inflammation, and aging [27]. In the mitochondrial matrix, peroxynitrite rapidly reacts with CO_2 to form the $ONOOCO_2^-$ adduct ($k = 6 \times 10^4\ M^{-1}s^{-1}$), which also participates in the nitration and oxidation of proteins and lipids. Formation of the adduct reduces the steady-state level of $ONOO^-$ from 30 nM to 2 nM [28] and is considered one of the pathways for detoxification of mitochondrial $ONOO^-$ at high mitochondrial CO_2 concentrations (1 mM).

Mitochondria have a multi-level system of enzymatic and non-enzymatic antioxidant defense, which ensures the detoxification of produced ROS. Enzymatic components include manganese-containing superoxide dismutase (MnSOD) [29], catalase, glutathione peroxidase, and phospholipid hydroperoxide glutathione peroxidase (which reduces hydroperoxides of phospholipids, lipoproteins, and cholesterol esters) [30], as well as enzymes involved in the regeneration of oxidized forms of small antioxidant molecules, such as glutathione reductase, thioredoxin reductase, glutaredoxin, and peroxiredoxin [31]. For the regeneration of glutathione-by-glutathione reductase and the restoration of thioredoxin 2 by thioredoxin reductase, the presence of NADPH is required. Mutations in the genes corresponding to these enzymes are associated with certain types of pathology, such as idiopathic cardiomyopathy, neurological diseases, impaired glucose metabolism, and tumors [32,33].

Non-enzymatic components of antioxidant defense include a number of hydrophilic and lipophilic free radical scavengers, such as cytochrome c, α-tocopherol, ascorbic acid, the reduced form of coenzyme Q10, and reduced glutathione (GSH).

The main part of mitochondrial $O_2^{\cdot -}$ diffuses into the matrix, where it interacts with intramitochondrial MnSOD, which catalyzes the reaction $_2O_2^{\cdot -} + 2H^+ \rightarrow O_2 + H_2O_2$. The steady-state concentrations of $O_2^{\cdot -}$ in the mitochondrial matrix are 0.2–0.3 nM at a content of 10–40 μM MnSOD reactive centers [31]. $O_2^{\cdot -}$ diffusing into the intermembrane space reacts with cytochrome c located on the outer side of the inner membrane and with Cu,Zn-SOD of the intermembrane space [34].

Glutathione peroxidase catalyzes the conversion of H_2O_2 and the reduction of lipid hydroperoxides (ROOH) with the participation of reduced glutathione [H_2O_2 (ROOH) + $_2$GSH → GSSG + $_2H_2O$ (ROH + H2O)] and is a unique enzyme that uses H_2O_2 as a substrate in the mitochondria of most mammalian organs, with the exception of the heart, where mitochondrial catalase has been described [35]. Due to the activity of glutathione peroxidase, 60% of H_2O_2 formed in the mitochondria is metabolized, indicating its important role in H2O2 detoxification and in the reduction of mitochondrial hydroperoxides [36]. GSH deficiency is associated with widespread mitochondrial dysfunction leading to cell damage [37]. The functioning of glutathione peroxidase requires a continuous supply of GSH to the mitochondria from the cytosol, where it is synthesized. A separate enzyme, NADPH-dependent glutathione reductase located in the mitochondrial matrix, is highly active and maintains the GSH/GSSG ratio in a highly reduced state [38].

Thus, ROS generation in structurally and functionally intact mitochondria is balanced by the antioxidant defense system, which limits the spread and bioavailability of ROS. A reduction in the level of antioxidant defense in mitochondria is a prerequisite for increased ROS production, the

development of oxidative stress, and subsequent mitochondrial dysfunction.

References

1. A Boveris, N Oshino, B Chance., (1972), The cellular production of hydrogen peroxide. 617-630.
2. A Boveris, B Chance., (1973), The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen.707-716.
3. Dionisi, T Galeotti, T Terranova, A Azzi., (1975), Superoxide radicals and hydrogen peroxide formation in mitochondria from normal and neoplastic tissues. 292-300.
4. Wang Wang, Huaqiang Fang, Linda Groom, Aiwu Cheng, Wanrui Zhang, [et al.], (2008), Superoxide flashes in single mitochondria. 279-290.
5. Hans Nohl, Lars Gille, Katrin Staniek., (2005), Intracellular generation of reactive oxygen species by mitochondria. 719-723.
6. Hans Nohl, Lars Gille, Katrin Staniek., (2004), The mystery of reactive oxygen species derived from cell respiration. 223-229.
7. J J Poderoso, C Lisdero, F Schöpfer, N Riobó, M C Carreras, et al., (1999), The regulation of mitochondrial oxygen uptake by redox reactions involving nitric oxide and ubiquinol. 37709-37716.
8. Shujuan Gao, Jun Chen, Sergey V Brodsky, Harer Huang, Stephen Adler, et al., (2004), Docking of endothelial nitric oxide synthase (eNOS) to the mitochondrial outer membrane: a pentabasic amino acid sequence in the autoinhibitory domain of eNOS targets a proteinase K-cleavable peptide on the cytoplasmic face of mitochondria. 15968-15974.
9. Katherine Green, Martin D Brand, Michael P Murphy., (2004), Prevention of mitochondrial oxidative damage as a therapeutic strategy in diabetes. S110-S118.
10. J.F. Turrens, A. Boveris., (1980), Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. 421-427.
11. A Boveris, E Cadenas., (2000), Mitochondrial production of hydrogen peroxide regulation by nitric oxide and the role of ubisemiquinone. 245-250.
12. Cadenas. E., A. Boveris., (1980), Enhancement of hydrogen peroxide formation by protophores and ionophores in antimycin-supplemented mitochondria. 31-37.
13. Ghafourifar. P, C. Richter., (1997), Nitric oxide synthase activity in mitochondria. 291-296.
14. GiuliviC., J.J. Poderoso, A. Boveris, (1998), Production of nitric oxide by mitochondria.11038-11043.
15. Brown.G.C., V. Borutaite, (2004), Inhibition of mitochondrial respiratory complex I by nitric oxide, peroxynitrite and S-nitrosothiols. 44-49.
16. F. Francesco Addabbo, Brian Ratliff, Hyeong-Cheon Park, Mei-Chuan Kuo, Zoltan Ungvari, et al., (2009), The Krebs cycle and mitochondrial mass are early victims of endothelial dysfunction: proteomic approach. 34-43.
17. David Z.I. Cherney, James W. Scholey, Joyce Zhou, Joseph Zimpelmann, Christopher Kennedy, et al., (2009), Endothelial nitric oxide synthase gene polymorphisms and the renal hemodynamic response to L-arginine. 327-332.
18. Elfering, S.L., T.M. Sarkela, C. Guillevin, (2002), Biochemistry of mitochondrial nitric-oxide synthase. 38079-38086.
19. Brookes, PS., (2004), Mitochondrial nitric oxide synthase.187-204.
20. A.J. Kanai, et al., (2001), Identification of a neuronal nitric oxide synthase in isolated cardiac mitochondria using electrochemical detection. 14126-14131.
21. Antunes, F., A. Boveris, E. Cadenas, (2004), On the mechanism and biology of cytochrome oxidase inhibition by nitric oxide.16774-16779.
22. J J Poderoso, M C Carreras, C Lisdero, N Riobó, F Schöpfer, et al., (1996), Nitric oxide inhibits electron transfer and increases superoxide radical production in rat heart mitochondria and submitochondrial particles. 85-92.
23. Rafael Radi, Adriana Cassina, Roberto Hodara, Celia Quijano, Laura Castro., (2002), Peroxynitrite reactions and formation in mitochondria. 1451-1464.
24. Valdez, L.B., T. Zaobornyj, A. Boveris, (2006), Mitochondrial metabolic states and membrane potential modulate mtNOS activity. 166-172.
25. Beckman, J.S., (1990), Ischaemic injury mediator. 27-28.
26. E Clementi, G C Brown, M Feelisch, S Moncada., (1998), Persistent inhibition of cell respiration by nitric oxide: crucial role of S-nitrosylation of mitochondrial complex I and protective action of glutathione. 7631-7636.
27. Beckman, K.B., B.N. Ames., (1998), The free radical theory of aging matures. 547-581.
28. M G Bonini, R Radi, G Ferrer-Sueta, A M Ferreira, O Augusto., (1999), Direct EPR detection of the carbonate radical anion produced from peroxynitrite and carbon dioxide. 10802-10806.
29. Fridovich, I., (1997), Superoxide anion radical (O2-.), superoxide dismutase's, and related matters.18515-18517.
30. T R Pushpa-Rekha, A L Burdsall, L M Oleksa, G M Chisolm, D M Driscoll., (1995), Rat phospholipid-hydroperoxide glutathione peroxidase. cDNA cloning and identification of multiple transcription and translation start sites. 26993-26999.
31. Maher, P., (2006), Redox control of neural function: background, mechanisms, and significance. 1941-1970.
32. Julie Turnbull, Hannes Lohi, Jennifer A Kearney, Guy A Rouleau, Antonio V Delgado-Escueta, et al., (2005), Sacred disease secrets revealed: the genetics of human epilepsy. 2491-2500.
33. A A Toye, J D Lippiat, P Proks, K Shimomura, L Bentley., et al., (2005), A genetic and physiological study of impaired glucose homeostasis control in C57BL/6J mice. 675-686.
34. Okado-Matsumoto, I. Fridovich, (2001), A. Subcellular distribution of superoxide dismutases (SOD) in rat liver: Cu,Zn-SOD in mitochondria. 38388-38393.
35. Radi, R. Turrens JF, Chang LY, Bush KM, Crapo JD, Freeman BA., (1991), Detection of catalase in rat heart mitochondria. 22028-22034.
36. K Nomura 1, H Imai, T Koumura, T Kobayashi, Y Nakagawa, (2000), Mitochondrial phospholipid hydroperoxide glutathione peroxidase inhibits the release of cytochrome c from mitochondria by suppressing the peroxidation of cardiolipin in hypoglycaemia-induced apoptosis. 183-193.
37. Meister, A. (1995), Mitochondrial changes associated with glutathione deficiency. 35-42.
38. Chance, B., H. Sies, A. Boveris., (1979), Hydroperoxide metabolism in mammalian organs. 527-605.

DOI:10.31579/2692-9406/267