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# Biochemistry of Reactive Oxygen and Nitrogen Species

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Additional information is available at the end of the chapter

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## Abstract

Reactive species or free radicals include reactive oxygen and nitrogen species that are called reactive oxygen nitrogen species. Reactive oxygen species are formed as a natural by-product of the normal metabolism of oxygen and have significant roles in cell signaling and homeostasis. The reactive oxygen species are generated as a by-product of biochemical reactions, in mitochondria, peroxisomes, cytochrome P450, and other cellular components. When oxygen homeostasis is not maintained, oxidative stress is increased in the cellular environment. Superoxide, hydrogen peroxide and hydroxyl radicals are normal metabolic by-products which are generated continuously by the mitochondria in growing cells. Microsomal cytochrome P450 enzymes, flavoprotein oxidases and peroxisomal enzymes are other significant intracellular sources of reactive oxygen species.

**Keywords:** Reactive oxygen species, mitochondria, NADPH oxidase, 5-lipoxygenase

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## 1. Introduction

All living aerobic multicellular organisms require molecular oxygen ( $O_2$ ) to survive rather than oxygen, which is susceptible to radical formation due to its electronic structure. Reactive oxygen species (ROS) are small molecules derived from oxygen molecules including free oxygen radicals, such as superoxide ( $O_2^{\cdot-}$ ), hydroxyl ( $\cdot OH$ ), peroxy ( $RO_2^{\cdot}$ ), and alkoxy ( $RO^{\cdot}$ ) as well as hypochlorous acid (HOCl), ozone ( $O_3$ ), singlet oxygen ( $^1O_2$ ), and hydrogen peroxide ( $H_2O_2$ ), which are non-radicals. These non-radicals are either oxidizing agents or easily

converted into radicals. Nitrogen-containing oxidants, such as nitric oxide ( $\text{NO}\cdot$ ) peroxynitrite ( $\text{ONOO}\cdot$ ), nitrogen dioxide ( $\text{NO}_2$ ) are called reactive nitrogen species (RNS) [1, 2].

Reactive species or free radicals include reactive oxygen and nitrogen species collectively and are called reactive oxygen nitrogen species (RONS). They are released from macrophages, neutrophils and dendritic cells in response to an inflammatory stimulus. RONS are highly reactive due to the presence of unpaired valence shell electrons or non-static bonds, and their proper regulation is vital for an efficient immune response and for limiting tissue damage [3].

Reactive oxygen species, chemically reactive molecules, containing oxygen, are formed as a natural by-product of the normal metabolism of oxygen and have significant roles in cell signaling and homeostasis. However, during times of environmental stress (e.g., UV radiation, heat exposure and ionizing radiation), their levels could increase dramatically. At high concentrations, ROS reacts readily with lipids, proteins, carbohydrates, and nucleic acids. This may result in significant damage to cell structures, and cumulates into a situation known as oxidative stress [4, 5]. Oxidative stress is a condition when the balance between the production of oxidants and their removal by antioxidants gets disturbed leading to increased production and accumulation of oxidants in the body. Aging, chronic inflammatory diseases, smoking, diabetes neurodegenerative diseases, cancer, etc., lead to generation of oxidative stress [3].

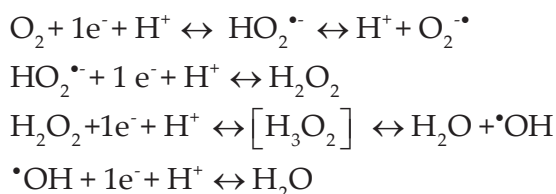
## 2. Types of free radicals

Free radicals are a group of highly reactive chemical molecules with one or more unpaired electrons. These substances are capable of giving rise to chain reactions involving a number of steps, each of which forms a free radical that triggers the next step. Initiation, propagation and termination are three phases of these reactions. There are some different types of free radicals. Oxygen-centered, nitrogen-centered, carbon-centered and sulphur-centered radicals are different free radical species [6, 7].

### 2.1. Oxygen-centered radicals

In living system oxygen radicals represent the most important class of radical species. In biochemistry, the free radicals are often referred as ROS, because biologically, the most significant free radicals are oxygen-centered [8, 9].

Radical formation is realized through subsequent steps [8].



Due to oxygen reduction, three important intermediate products are generated: superoxide anion, hydrogen peroxide and hydroxyl radical.

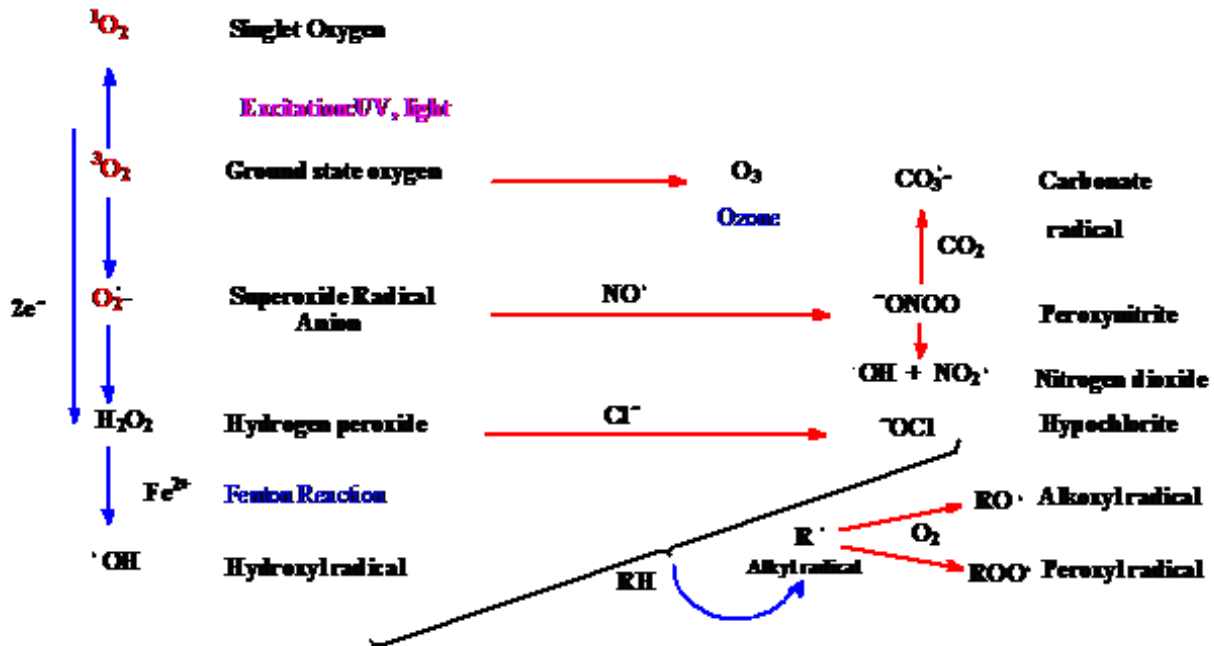
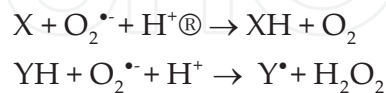


Figure 1. Main reactive oxygen species

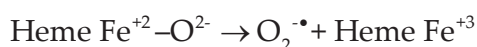
### 2.1.1. Superoxide anion

Superoxide anion ( $\text{O}_2^{\bullet-}$ ), the most common ROS, is generated in mitochondria, in cardiovascular system and other parts of the body [3, 10]. The electron transport chain (ETC) is responsible for most of the superoxide generation through partial reduction of oxygen [11].

Radical formation is possible:



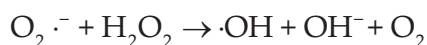
In aerobic organism most of the oxygen is reduced to water in mitochondrial respiratory chain. However, a small proportion of the oxygen molecules (1% -2%) is converted to superoxide anion radical. These reactions occur in respiratory chain by complex I (NADH: ubiquinone oxidoreductase) and complex III (ubiquinol: cytochrome *c* oxidoreductase) [12]. Another important pathway to form superoxide is represented by heme oxidation. The iron (of heme group) is reduced to ferrous (Fe II) in the deoxyhemoglobin and when it attaches to oxygen an intermediate structure is formed [8].



In addition to this, dihydrorotate dehydrogenase, aldehyde oxidase, and xanthine oxidase are oxidative enzymes that can also produce superoxide anion.

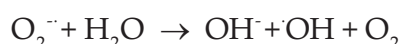
### 2.1.2. Hydrogen peroxide

Hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) is a neutral molecule, which is the least reactive molecule among ROS and is stable under physiological pH and temperature in the absence of metal ions. It is highly diffusible and does cross the plasma membrane easily.  $\text{H}_2\text{O}_2$  is formed in our body by large number of reactions and yields potent species [3].  $\text{H}_2\text{O}_2$  could be produced from superoxide anion by superoxide dismutase (SOD) through a dismutation reaction. Amino acid oxidase and xanthine oxidase can also produce  $\text{H}_2\text{O}_2$  from superoxide anion. In the presence of metal ions and superoxide anion  $\text{H}_2\text{O}_2$  can produce the hydroxyl radical [13, 15].

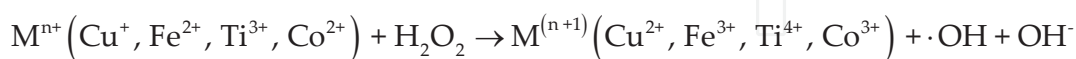


### 2.1.3. Hydroxyl radical

Hydroxyl radical ( $\cdot\text{OH}$ ), the most reactive and dangerous radical, can be formed from superoxide anion and  $\text{H}_2\text{O}_2$  in the presence of metal ions, and it has a very short *in vivo* half-life of about  $10^{-9}$ s [13]. As a result, when produced *in vivo*, OH reacts close to its site of formation. Most of hydroxyl radicals are produced in the excess of superoxide anion and  $\text{H}_2\text{O}_2$  by Haber-Weiss reaction [8, 14].



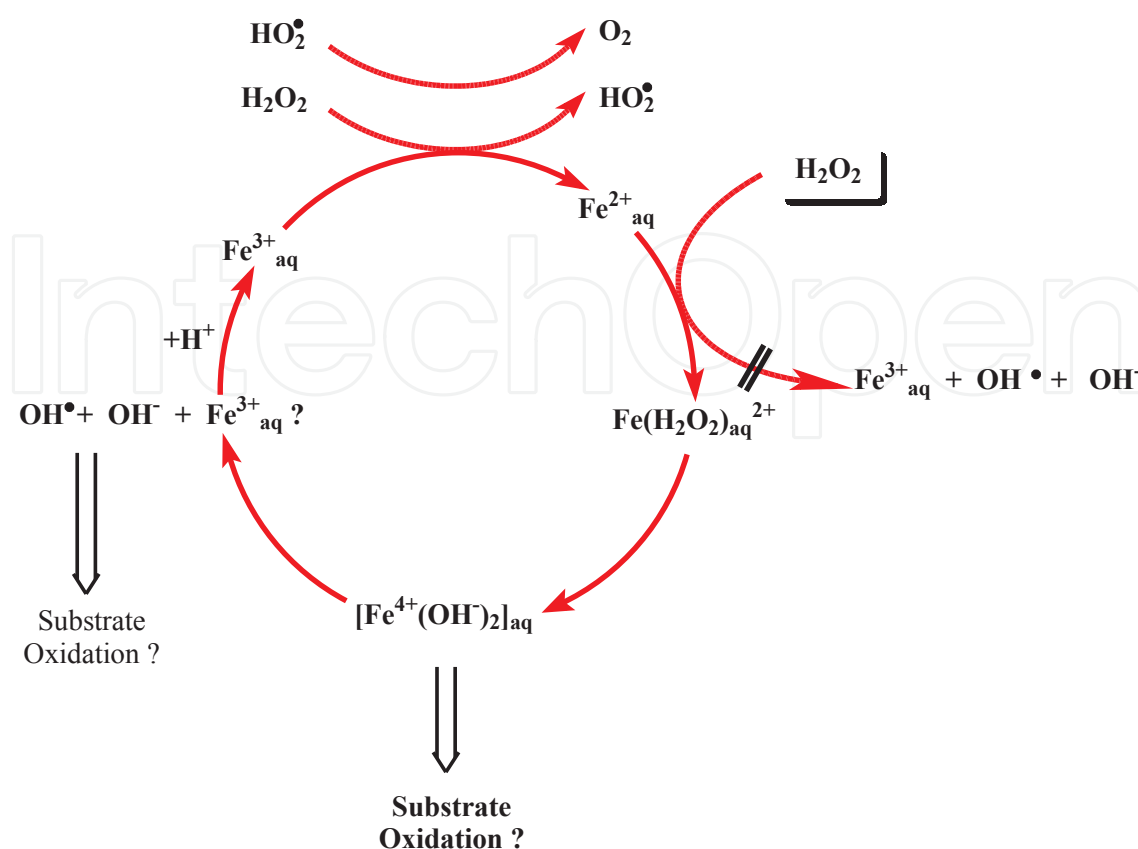
It can also be produced by multiple pathways; such as, decomposition of water because of ionizing radiation that form hydroxyl radicals and hydrogen atoms, and photolytic decomposition of alkyl hydroperoxides. *In vivo*, primarily hydroxyl radicals emerge from the metal-catalyzed breakdown of  $\text{H}_2\text{O}_2$ , through *Fenton reaction* [8, 14].



The Fenton reaction is generally considered to yield the  $\cdot\text{OH}$ ; following as shown from spin-trapping and hydroxylation [15].

### 2.1.4. Singlet oxygen

Singlet oxygen ( $^1\text{O}_2$ ), a non-radical, can be generated by an input of energy that rearranges the electrons, and it is rather mild and nontoxic for mammalian tissue. It is formed during



**Figure 2.** Mechanistic presentation of possible reactions involved in the thermal Fenton reaction with simplified notations used for the various iron complexes

photosensitization and chemical reactions [3]. In the human beings,  $^1\text{O}_2$  is both a signal and a weapon, with therapeutic potency against various pathogens such as microbes, viruses, and cancer cells. Two different pathways in biology can produce singlet oxygen.  $^1\text{O}_2$  can directly oxidize proteins, DNA, and lipids and has been known to be involved in cholesterol oxidation that can participate in *Diels-Alder* reactions. It can be generated by chemical processes, such as spontaneous decomposition of hydrogen trioxide in water or the reaction of  $\text{H}_2\text{O}_2$  with hypochlorite.  $^1\text{O}_2$  reacts with an alkene ( $-\text{C}=\text{C}-\text{CH}_2-$ ) by abstraction of the allylic proton in an ene-type reaction to the allyl hydroperoxide  $\text{HO}-\text{O}-\text{R}$  ( $\text{R} = \text{alkyl}$ ), which can then be reduced to the allyl alcohol [16].

## 2.2. Carbon-centered radicals

Carbon centered radicals are described as follows.

### 2.2.1. Peroxyl and alkoxy radicals

Peroxyl ( $\text{ROO}^\bullet$ ) and alkoxy ( $\text{RO}^\bullet$ ) radicals are good oxidizing agents, having more than 1000 mV of standard reduction potential. Irradiation of UV light or the presence of transition metal ions can cause hemolysis of peroxides to produce peroxyl and alkoxy radicals.



Aromatic alkoxy and peroxy radicals are less than respective open chain radicals because of the delocalization of electrons in the ring. ROO is reactive and formed from lipids, proteins, DNA and carbohydrates during oxidative damage [3]. Peroxy radicals are generated by a direct reaction of oxygen with alkyl radicals (R) [13].

### 2.3. Nitrogen-centered free radicals

NO and its by-products, such as nitrate ( $\text{NO}_3^-$ ), nitrite ( $\text{NO}_2^-$ ), peroxynitrite ( $\text{ONOO}^-$ ), and 3-nitrotyrosine are called RNS. More recently the role of the RNS has been shown to have a direct role in cellular signaling, vasodilatation, and immune response [10].

#### 2.3.1. Nitric oxide

NO is generated during the breakdown of arginine to citrulline by a family of NADPH-dependent enzymes called nitric oxide synthase [10, 11]. It is an uncharged lipophilic molecule containing a single unpaired electron, which causes it to be reactive with other molecules such as oxygen, superoxide radicals and glutathion. While NO is not a very reactive free radical, it is able to form other reactive intermediates, which have an effect on protein function and on the function of the entire organisms. These reactive intermediates can trigger nitrosative damage on biomolecules [10]. Therefore; NO could function as an oxidant or an antioxidant. NO is neurotransmitter and blood pressure regulator, and it can yield potent oxidants during pathologic states [3]. Overproduction of NO is involved in ischemia reperfusion, and neurodegenerative and chronic inflammatory diseases, such as rheumatoid arthritis and inflammatory bowel disease [3,13].

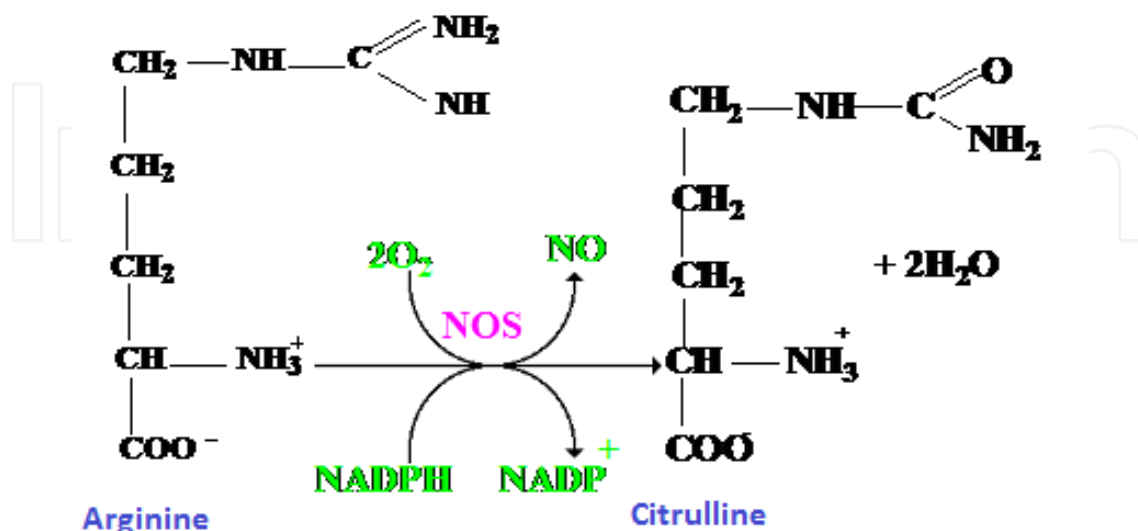


Figure 3. Nitric oxide formation

After NO is produced, it can inhibit cytochrome c oxidase. NO found in the mitochondria increases the production of ROS and RNS which can alter the activity of various processes such as mitochondrial biogenesis, respiration and oxidative stress [11].

NO is more stable and diffusible than hydroxyl radicals. These have shown hyperphosphorylation and inactivate retinoblastoma protein resulting in increased proliferation of human colon cancer cells. NO modifies DNA directly and inactivates the DNA repair enzymes. NO is very reactive signaling molecule and it is an important regulator for cellular functions. Nitrosative stress also plays a critical role in inflammation-associated carcinogenesis by activating a representative redox sensitive transcription factor. NO modifies DNA directly and inactivates the DNA repair enzymes [3]. NO is exposed to human blood plasma which can deplete the concentration of ascorbic acid, uric acid, and initiate lipid peroxidation.

### 2.3.2. Peroxynitrite

NO reacts with  $O_2^-$  with a high rate constant to give peroxynitrite ( $O_2^- + NO \rightarrow OONO^-$ ) [15, 17], which may spontaneously decompose to yield  $NO_2^-$  and hydroxyl radical ( $\cdot OH$ ) [18]. At physiological pH,  $ONOO^-$  is a stronger oxidant than  $O_2^-$  or NO, and it oxidizes lipids, proteins, nitrated amino acids and DNA [19, 20].

Peroxynitrite is a cytotoxic and causes tissue injury and oxidizes low-density lipoprotein. The significance of  $OONO^-$  as a biological oxidant comes from its high diffusibility across cell membranes.  $OONO^-$  appears to be an important tissue-damaging species generated at the sites of inflammation and has been shown to be involved in various neurodegenerative disorders and several kidney diseases. The molecule can cause direct protein oxidation and DNA base oxidation and modification acting as a "hydroxyl radical-like" oxidant. Nitrotyrosine, which can be formed from peroxynitrite-mediated reactions with amino acids, has been found in age-associated tissues [9, 13].

Peroxynitrite as a potential biomarker of inflammation-associated cancers and a product formed by a reaction between NO radical and superoxide anion, causes DNA damage by generating 8-nitroguanine. Thus, oxidative and nitrosative DNA damage products have been implicated in the initiation of inflammation-driven carcinogenesis [3].

### 2.3.3. Nitrogen dioxide

Nitrogen dioxide ( $NO_2\cdot$ ) is formed from the reaction of peroxy radical and NO.  $NO_2\cdot$  initiates lipid peroxidation for production of free radicals and also oxidizes ascorbic acid [13].  $NO_2\cdot$ , a major decomposition product of NO and very reactive oxidant, is able to oxidize tyrosine to 3-nitrotyrosine. Moreover,  $NO_2\cdot$  is a substrate for the mammalian peroxidase and lactoperoxidase and forms  $NO_2\cdot$  via peroxidase-catalyzed oxidation of  $NO_2\cdot$ . This provides an additional pathway contributing to cytotoxicity or host defense associated with increased NO production and an alternative pathway for the formation of 3-nitrotyrosine [10].



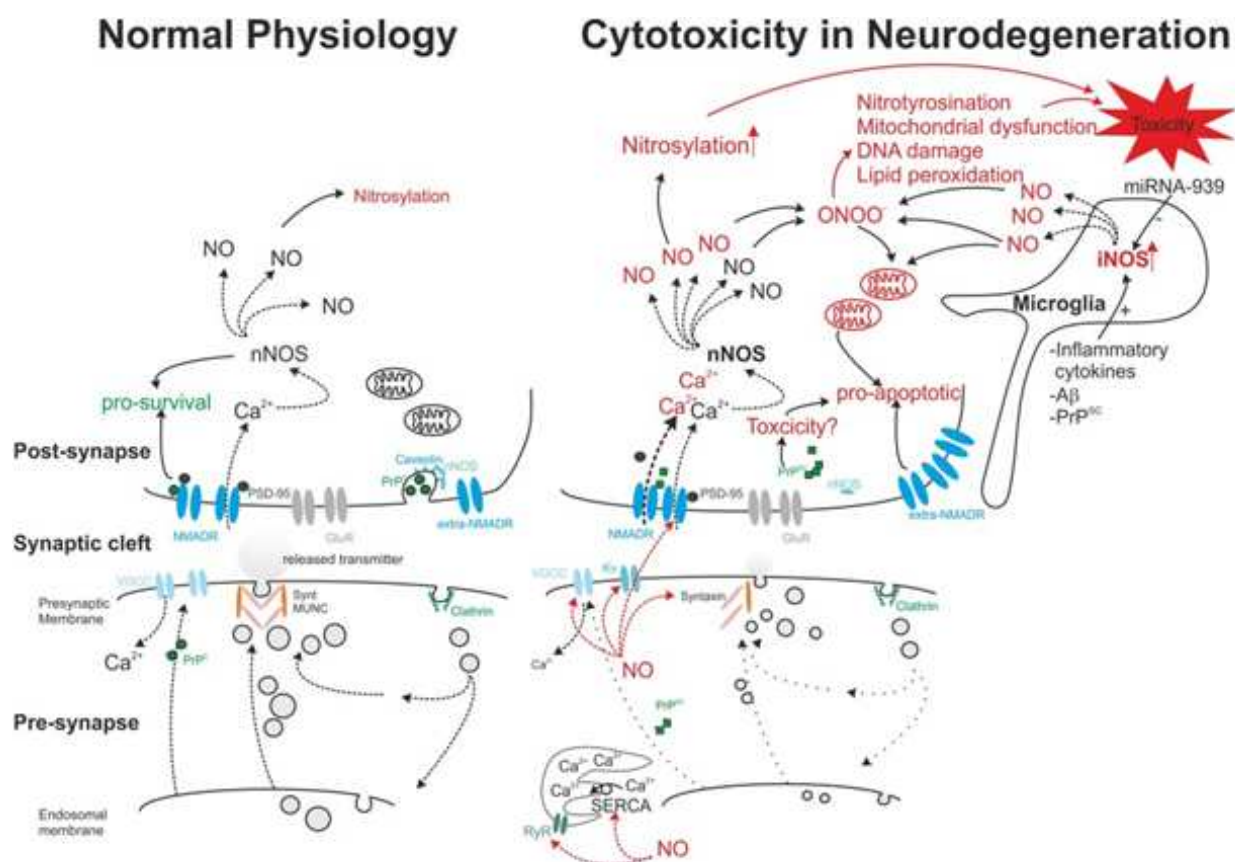


Figure 4. Nitric oxide and cytotoxicity

### 3. Biochemistry of ROS and RNS

The generation of ROS can occur as a product of biochemical reactions, in mitochondria, peroxisomes, cytochrome P450, and other cellular components [21, 22]. ROS are generated mainly by the mitochondrial ETC. Almost all cells, and tissues continuously convert a small proportion of molecular oxygen to ROS in ETC. ROS produced by other pathways, including the respiratory burst taking place in activated phagocytes, are ionizing the damaging effect of the radiation on components of cell membranes, and by-products of several cellular enzymes (NADPH oxidases, xanthine oxidase, nitric oxide synthase) [23]. The formation of ROS is a natural consequence of aerobic metabolism and is integral for maintaining tissue oxygen homeostasis. When oxygen homeostasis is not maintained, there is an increase in oxidative stress in the cellular environment. Superoxide, hydrogen peroxide and hydroxyl radicals are normal metabolic by-products which are generated continuously by the mitochondria in growing cells. Microsomal cytochrome P450 enzymes, flavoprotein oxidases and peroxisomal enzymes are other significant intracellular sources of ROS [24].

ROS play the key roles in both health and disease. ROS also have an important role in several physiologic processes such as normal vascular cell functioning and maintaining vascular

diameter regulation. ROS carry out this function by mounting effective immune response, acting as possible signaling molecules and regulating glucose uptake by skeletal muscle [3]. They have a role in response to growth factor stimulation and control of inflammatory responses. They participate in the regulation of differentiation, proliferation, growth, apoptosis, cytoskeletal regulation, migration, and contraction [4].

ROS contributes to a wide range of pathologies and many of the implicated diseases which lead to death, such as chronic inflammation and autoimmune diseases (diabetes, rheumatoid arthritis, lupus), sensory impairment (ocular disease, hearing loss), cardiovascular diseases (atherosclerosis, hypertension, ischemia/reperfusion injury), cancer (breast, renal, lung), fibrotic disease (pulmonary and liver fibrosis, diabetic nephropathy), obesity, insulin resistance, neurological disorders (Parkinson's, Alzheimer's, ALS, schizophrenia), and infectious diseases (septic shock, influenza, hepatitis, HIV) [3, 4].

ROS generation is generally a cascade of reactions which starts with the production of superoxide. Superoxide rapidly dismutates to hydrogen peroxide spontaneously, particularly at low pH or is catalyzed by SOD. The other generations include the reaction of superoxide with NO to form peroxynitrite, the peroxidase-catalyzed formation of hypochlorous acid from hydrogen peroxide, and the iron-catalyzed Fenton reaction leading to the generation of hydroxyl radical [2,25].

ROS act with a large number of biomolecules (proteins, lipids, carbohydrates, and nucleic acids). ROS may irreversibly destroys and alter the function of these molecules after interacting with them. As a result, they have been increasingly identified in biological organisms as major contributors to damage. Harman has showed the role of ROS in the aging process [26], after these ROS become cell damaging agents in aging theory. ROS also have an important role in host defense because ROS generation deficiencies reduce the killing ability of leukocytes. However, over the last decades, a second important concept of ROS have been evolving. Furthermore, ROS have a reversible regulatory process in virtually all cells and tissues [27].

### 3.1. Biological sources of ROS

In mammalian cells the biological sources of ROS are as follows; mitochondria [28], endoplasmic reticulum [29], peroxisomes [12], cytosol [30], plasma membrane [31] and extracellular space [32]. Major sources of ROS include metabolic processes and cellular respiration processes. During the metabolic processes the peroxisome catabolizes biomolecules that remove hydrogen in an oxidative reaction creating  $H_2O_2$ . While the cellular respiration oxygen is reduced the intermediates with odd electrons can escape the chain.

Typically the initial reaction is an electron transfer of oxygen to  $O_2^-$ , which then dismutates to  $H_2O_2$ .  $O_2^-$  does not readily cross membranes, and is short-lived and local in its effects, but SOD converts  $O_2$  to longer-lasting and membrane-diffusible  $H_2O_2$ . When  $O_2$  react with NO, the highly reactive ONOO is formed. Peroxidases catalyze reactions involving  $H_2O_2$ , resulting in the generation of HOCl and  $^1O_2$  among other species. Finally the Haber-Weiss reaction uses an iron ion catalyst to generate hydroxyl radicals from  $O_2^-$  and  $H_2O_2$ . Major sources of ROS

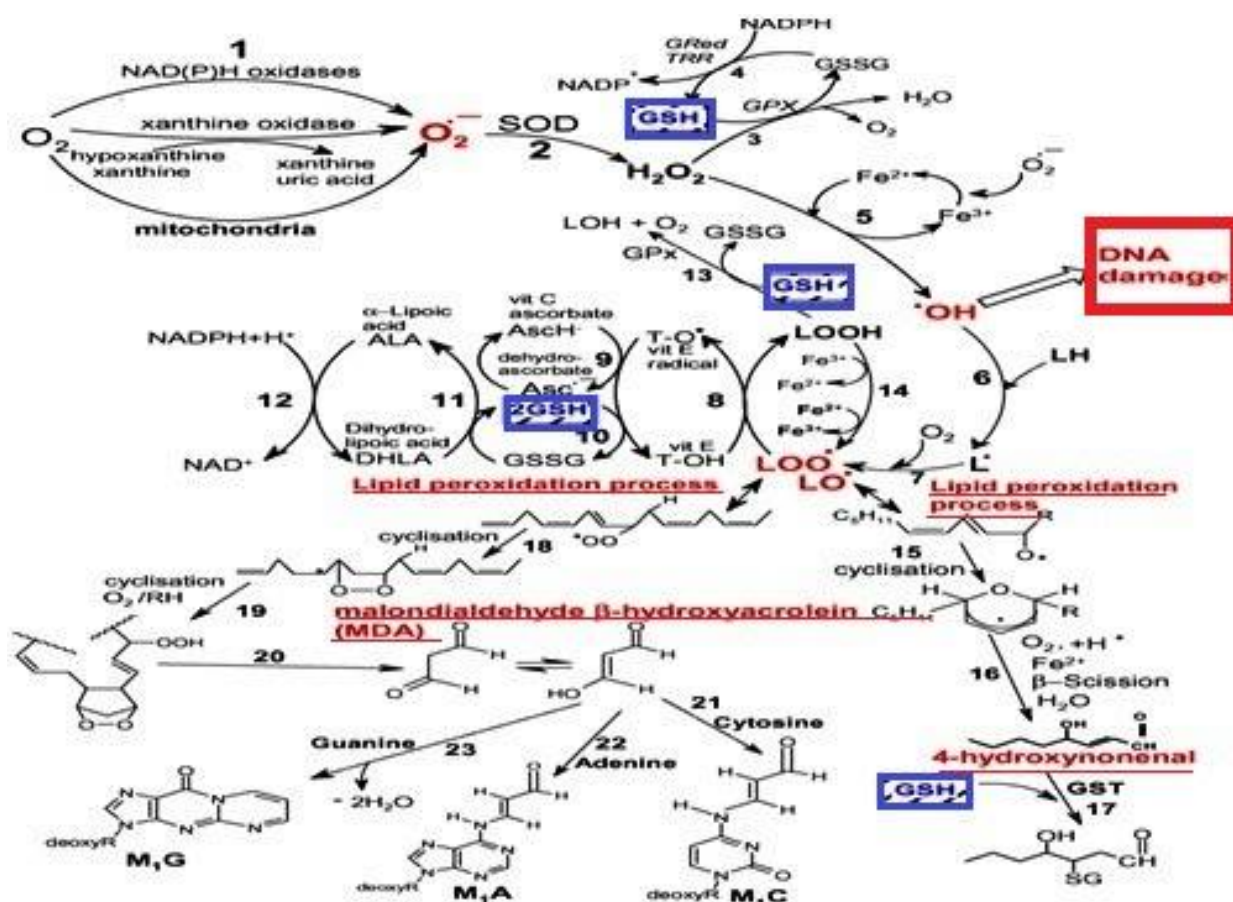


Figure 5. Pathways of ROS formation

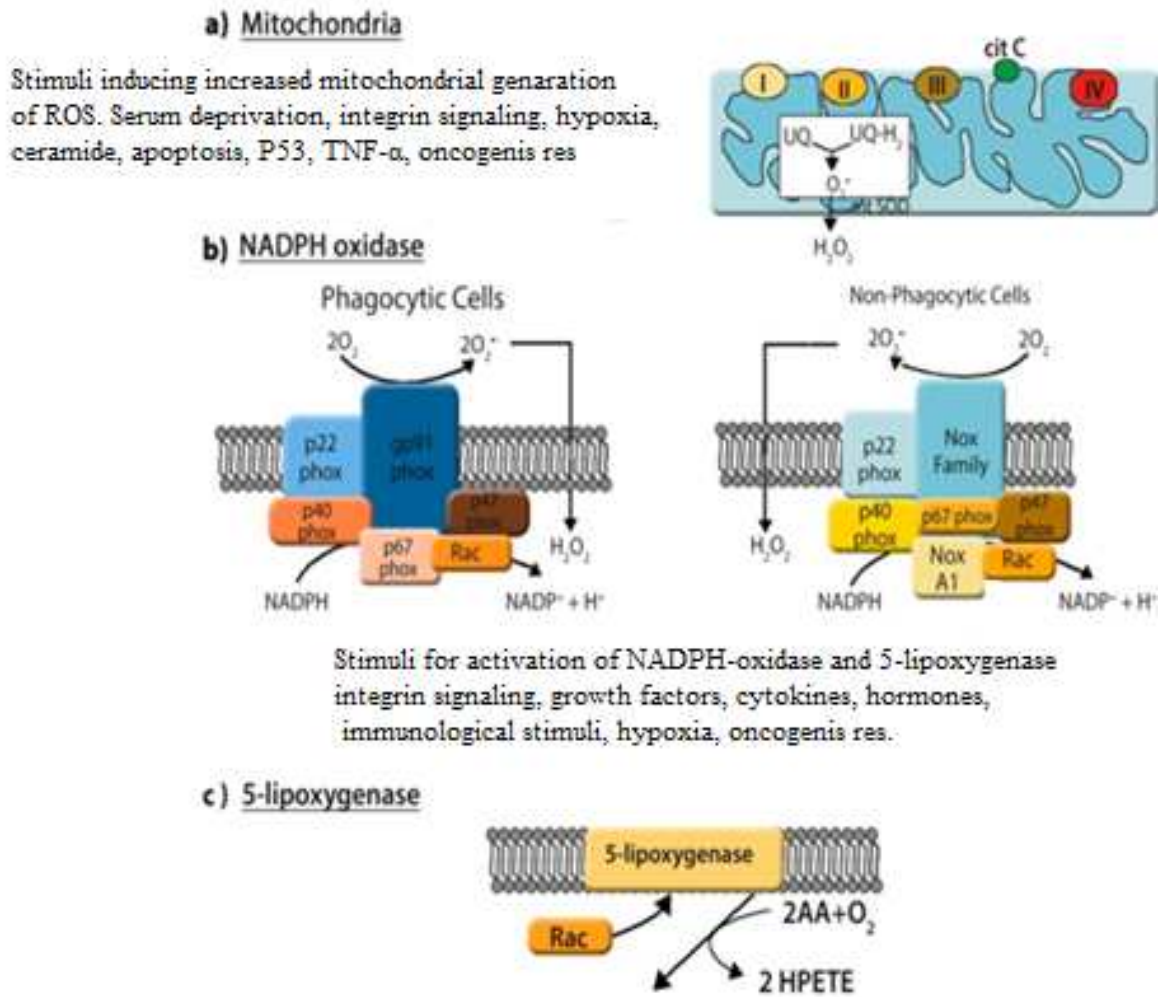
includes cellular respiration and metabolic processes, though ROS may also be generated by radiation [4].

### 3.2. Biosynthesis pathway of ROS

Mitochondria, NADPH oxidase and 5-lipoxygenase (5-LOX) are three major sources of ROS formation.

#### 3.2.1. Mitochondrial ROS production

The generation of mitochondrial ROS is the result of oxidative phosphorylation. In cytochrome chain, electrons derived from FADH or NADH can directly react with oxygen or other electron acceptors, and generate free radicals [3]. ROS, as generated by mitochondria during respiration, induce oxidative stress, which accumulates over life and is considered as the proximal mechanism of aging and a major determinant of degenerative disease, including cancer, and lifespan (the free radical or mitochondrial theory of aging). Recent data showed that specific ROS in particular  $H_2O_2$  is directly implicated in the physiological regulation of different signal transduction pathways, including the insulin/IGF<sub>1</sub> pathway. Indeed mitochondrial ROS



**Figure 6.** The major pathway formation of ROS

production has been found to contribute to the regulation of several cellular processes in a specific manner. A role for ROS as signaling molecules is further supported by recent findings that the generation of  $H_2O_2$  by mitochondria is not only as a by-product of respiration but also as a result of specific enzymatic systems, such as p66Shc. P66Shc functions as an inducible redox enzyme, which is activated by stress and triggers apoptosis to regulate signal transduction and transcription. Regardless of the purpose p66Shc utilizes to shift the intracellular redox balance towards oxidation, it appears that mitochondrial ROS formation ability is evolved to set intracellular ROS levels. Therefore; one could hypothesize that the mitochondrial-mediated oxidative stress may have a critical oncogenic role when tumor suppressor mechanisms decreased rather than acting as primary mutagens [33-35].

Mitochondria are responsible for 90% of the energy production in cells, and thus tissues, organs and the body as at the whole needs to function. Hence, they are known as the “powerhouse of a cell”: the core of cellular energy metabolism, being the site of most ATP generation through mitochondrial oxidative phosphorylation [36]. In this process, electrons liberated from reducing substrates are delivered to  $O_2$  establishing an electrochemical gradient used to drive

ATP synthesis. During the oxidative phosphorylation, the reduction of oxygen by one electron at a time ( $O_2 \rightarrow O_2^{\cdot-} \rightarrow H_2O_2 \rightarrow \cdot OH \rightarrow H_2O$ ) produces ROS, relatively stable intermediates with one unpaired electron [37]. There is a lot of evidence supporting the point of view that mitochondria are not a major source of ROS in the cell. In fact, about 90% to 95% of cellular oxygen is used up in oxidative phosphorylation and 3% from that pool can be converted to superoxide which is a very strong argument to mitochondria as a main source of this oxygen radical. Consequently, in mammalian cells mitochondria are the major source of ROS [38].

The primary ROS are generated by mitochondria, as a result of monoelectronic reduction of  $O_2$  which is the precursor of most ROS and a mediator in oxidative chain reactions.  $O_2$  is produced in mammalian cells enzymatically by NADPH oxidase, cytochrome P450-dependent oxygenases and xanthine oxidase as well as in course of a single electron is directly transferred to  $O_2$ . Furthermore,  $O_2$  can produce RNS which react with other radicals, such as NO.

Hydrogen peroxide is produced by dismutation of  $O_2$ , catalyzed by SODs, whether spontaneously or through a reaction in mitochondria. Because  $H_2O_2$  is relatively stable and membrane permeable, it can diffuse within the cell and be eliminated by cytosolic or mitochondrial antioxidant systems such as catalase, glutathione peroxidase, and thioredoxin peroxidase. Mitochondrial generated  $H_2O_2$  can also act as a signaling molecule in the cytosol, affecting multiple networks which control, for instance, cell cycle, stress response, energy metabolism, and redox balance [39, 40].

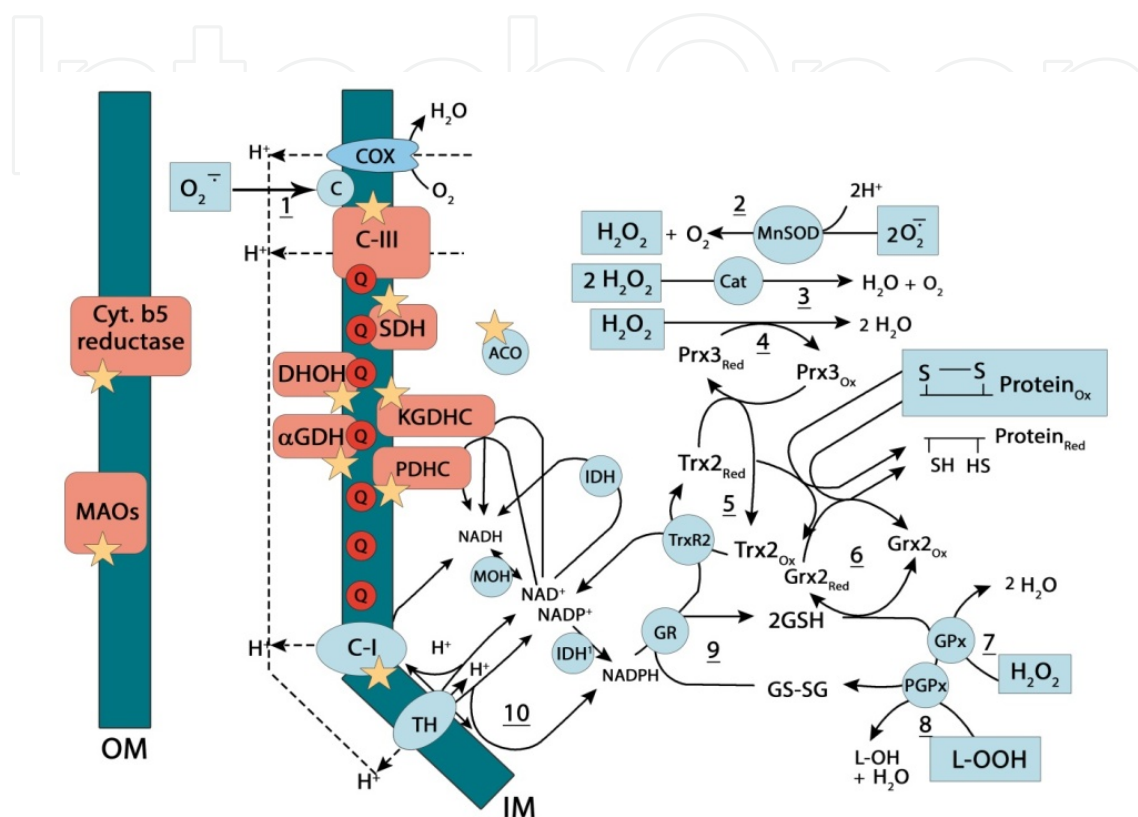
Hydroxyl radical ( $\cdot OH$ ) can be formed in the presence of metal ions by Fenton reaction when  $H_2O_2$  is not metabolized. As one of the strongest oxidants hydroxyl radical is highly reactive and may damage the other molecules. Therefore, mitochondria have developed an efficient  $H_2O_2$  removal systems. These metal-chelating mechanisms include chaperone proteins and prevent the formation of this radical. Even if there are at least ten enzymes in mammalian mitochondrial for ROS production, their capacity to produce ROS greatly differ in a tissue-specific manner. *In vitro* experiments have demonstrated that  $H_2O_2$  generation is both substrate specific and organ specific [41, 42].

Mitochondrial ROS are a product of respiration and generally occur at the ETC. As a terminal component of the ETC, cytochrome c oxidase (Complex IV) receives four electrons from cytochrome c and reduces one  $O_2$  molecule to two  $H_2O$ . The first mitochondrial site producing ROS was identified at the Complex III located at the inner side of inner mitochondrial membrane (bc1 complex, ubiquinone: cytochrome c reductase). The primary ROS produced at this site are  $O_2^{\cdot-}$ , through the referred Q-cycle [43].

Unsaturated fatty acids in mitochondrial membranes, which are components of phospholipids, can be easily oxidized by the hydroxyl radical. Lipid 4-hydroxynonenal (4-HNE), malondialdehyde (MDA) and acrolein are the lipid peroxidation products. Generation of these lipid radicals lead to the production of a new radical, which causes various effects in the cell such as affecting membrane fluidity and increasing permeability [44]. Lipid radicals can covalently modify membrane proteins as well as cause "lipoxidative" damage to the mtDNA because they diffuse easily in the membranes [45]. For instance, 4-hydroxy 2-nonenal inhibits

adenine nucleotide translocase activity in mitochondria on account of the modification of sulfhydryl groups in adenine nucleotide translocase [46].

The nine known sources of ROS in mitochondria are shown in Fig. 6 in the context of location within a mitochondrion. ROS producing seven enzymes will be discussed in the following text.



**Figure 7.** Sources of ROS in mitochondria

1. **Complex III** (cytochrome c oxidoreductase, EC 1.10.2.2) enzyme is located closer to the outer and the inner coupling membrane and widely distributed in mammalian tissues. Complex III produces superoxide at both site of membrane. The enzyme oxidizes cytoplasmic NAD(P)H and reduces cytochrome *b*5 in the outer membrane. It may also reduce ascorbyl free radical and, therefore, be involved in regeneration of ascorbate in mammalian liver. The enzyme is upregulated in the patients suffering from schizophrenia, thus implying a role in the etiology of the disease. There is a single report that mitochondrial cytochrome *b*5 reductase may produce superoxide with a high rate of ~300 nmol/ min per mg protein [36, 47].
2. **Monoamine oxidase** (MAO, EC 1.4.3.4) is located in the outer mitochondrial membrane and contain flavin adenine dinucleotide (FAD). Two isoforms MAO-A and MAO-B are identified with their specificity and sensitivity to inhibitors. Norepinephrine and serotonin are oxidized by MAO-A, whereas the substrates for MAO-B are phenylethylamine and benzylamine. This enzyme is found in all the tissues such as, lung, liver blood vessels,

heart, the nervous system, etc. The enzyme catalyze oxidation of biogenic amines and produce toxic molecules, aldehydes, ammonium hydroxide and hydrogen peroxide. MAO cause development of oxidative stress in heart and nervous system. MAO is also a major source of  $H_2O_2$  in ischemia [48] and aging [42]. Furthermore MAO gives rise to peroxidation of phospholipids in mitochondria. The inhibitors of MAO decreased production of hydrogen peroxide and thus protect the heart [43].

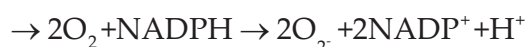
3. **Dihydroorotate dehydrogenase** (DODH, dihydroorotate: ubiquinone oxidoreductase, EC 1.3.99.11) is a flavoprotein located in the outer surface of inner mammalian mitochondrial membrane. The enzyme catalyzes the conversion of dihydroorotate to orotate, which is a step in the *de novo* synthesis of uridine monophosphate. DODH has been found in many cells, such as tumor, mucosal, ileum, colon, crypts, kidney cortex, heart and liver. The most active enzyme is found in heart and liver mitochondria. The enzyme contains flavin mononucleotide (FMN) containing active site as a source of superoxide. Superoxide is formed during aerobic oxidation of dihydroorotate in the presence of cyanide. However, histochemical studies showed that heart and kidney cortex mitochondria produce hydrogen peroxide during dihydroorotate oxidation. Drugs specifically interacts with the hydrophobic channel of the enzyme by preventing FMN-ubiquinone oxydoreduction thus decreasing ROS formation in cancer cell cultures.
4. **Mitochondrial glycerophosphate dehydrogenase** (mGPDH,  $\alpha$ -glycerol-3-phosphate: ubiquinone oxidoreductase, EC 1.1.99.5) is a FAD-linked enzyme and located in the outer surface of inner mitochondrial membrane. The enzyme catalyze oxidation of glycerol-3-phosphate to dihydroxyacetone phosphate by utilizing mitochondrial coenzyme Q as electron acceptor. The mGPDH has influence on lipid metabolism and catalyze transfer of reducing equivalents from cytosolic NADH to the respiratory chain in mitochondria, which is so-called glycerol phosphate shuttle. Its activity and content substantially vary in different tissues. Placenta, brown adipose tissue, testis, skeletal muscle, langerhans and brain tissue have high mGPDH activity, whereas heart, liver and kidney mitochondria have low activity. In heart mitochondria the ratio of mGPDH to succinate dehydrogenase is approximately 1/15 [40, 49]. Thyroid hormones increase its activity. ROS production is occurred during  $\alpha$ -glycerophosphate oxidation. These productions are increased by the inhibitors of complex III, antimycin A and myxothiazol.
5. **Complex II** [succinate dehydrogenase complex (SDH, succinate:ubiquinone oxidoreductase, EC1.3.99.1)] is a marker enzyme located at the inner surface of inner mitochondrial membrane. The enzyme oxidizes succinate to fumarate, which is one of reactions in Krebs cycle by using coenzyme Q as an electron acceptor. Although oxidation of succinate by good-quality mitochondria from most mammalian tissues can produce ROS with a high rate, the source of ROS is Complex I, not SDH. The mechanism involves reverse electron transfer from SDH-reduced coenzyme Q to Complex I. Nevertheless, isolated SHD reconstructed in liposomes can produce ROS by itself. Authors concluded that reduced FAD of SDH generates ROS in the absence of its electron acceptor. There is also a report implying that SDH can generate ROS in submitochondrial particles. However; the conclusion was based solely on the inhibition of ROS production by carboxin, a specific

inhibitor of SDH. The same inhibitor also suppressed antimycin-induced ROS production and ROS production supported by NADH oxidation. The former is thought to originate from Complex III that is not inhibited by carboxin whereas the effect of carboxin on NADH-supported ROS production may not be readily explained either. Therefore, it is unclear whether SDH produces ROS *in situ*, in mitochondria [43].

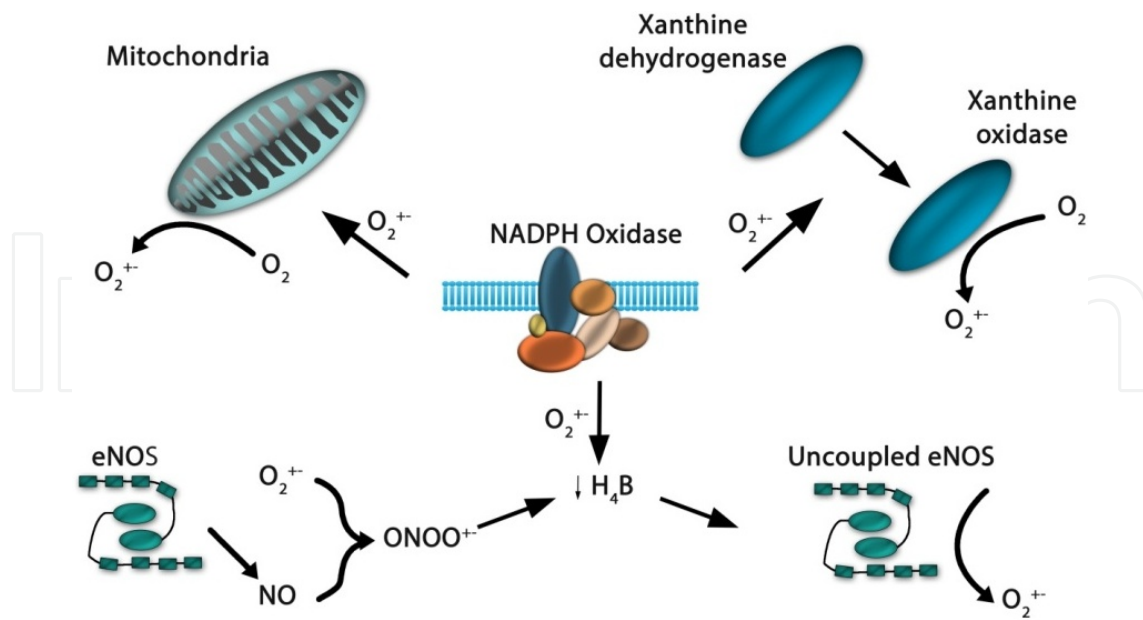
6. **Mitochondrial aconitase** (m-aconitase, EC 4.2.1.3) is an enzyme localized to the matrix space of mitochondria; it participates in tricarboxylic acid cycle catalyzing a conversion of citrate to isocitrate. The enzyme contains an iron-sulfur cluster that can be oxidized by superoxide, inactivating m-aconitase. Recently, it was found that isolated aconitase oxidized by either superoxide or hydrogen peroxide produces hydroxyl radical. Vasquez-Vivar et al. [50] state that similar continuous hydroxyl radical production may occur upon superoxide-driven redox-cycling of aconitase in mitochondria.
7. **Ketoglutarate dehydrogenase complex** (KGDHC, 2-oxoglutarate dehydrogenase) is an integral mitochondrial enzyme tightly bound to the inner mitochondrial membrane on the matrix side. In the tricarboxylic acid cycle, it catalyzes the oxidation of  $\alpha$ -ketoglutarate to succinyl-CoA using  $\text{NAD}^+$  as electron acceptor. Structurally, KGDHC is composed of multiple copies of three enzymes:  $\alpha$ -ketoglutarate dehydrogenase, dihydrolipoamide succinyltransferase, and lipoamide dehydrogenase. The E3 component of KGDHC is a flavin-containing enzyme; it is identical to the E3 component of another integral mitochondrial enzyme located in the matrix, pyruvate dehydrogenase (PDHC). The E3 component is also known as dihydrolipoamide dehydrogenase, which is ubiquitously present in mammalian mitochondria. Both PDHC and KGDHC can generate superoxide and hydrogen peroxide; ROS production was shown with isolated purified enzymes from bovine heart and in isolated mitochondria. The source of ROS in KGDHC appears to be the dihydrolipoamide dehydrogenase component. Earlier, isolated dihydrolipoamide dehydrogenase was shown to produce ROS. In mitochondria and with isolated enzyme, ROS production from KGDHC was stimulated by a decrease in availability of its natural electron acceptor,  $\text{NAD}^+$  [51, 52].

### 3.2.2. NADPH oxidase

In aerobic organisms, electrons are transferred from the reductant to the oxidant. These are always catalyzed by oxidoreductases that give rise to superoxide production the primary ROS molecule in biological systems. In mammalian cells, cyclooxygenase, lipoxygenase, cytochrome P450 enzymes, nitric oxide synthase xanthine oxidase, mitochondrial NADH:ubiquinone oxidoreductase (complex I) and NADPH oxidase have been identified as potential sources of superoxide. Contrary to the other oxidoreductase, the NADPH oxidase is the major enzymatic source of ROS generation in cells but the other enzymes produce ROS only as by-products. It catalyzes the production of superoxide by the reduction of oxygen, using NADPH as the electron donor.

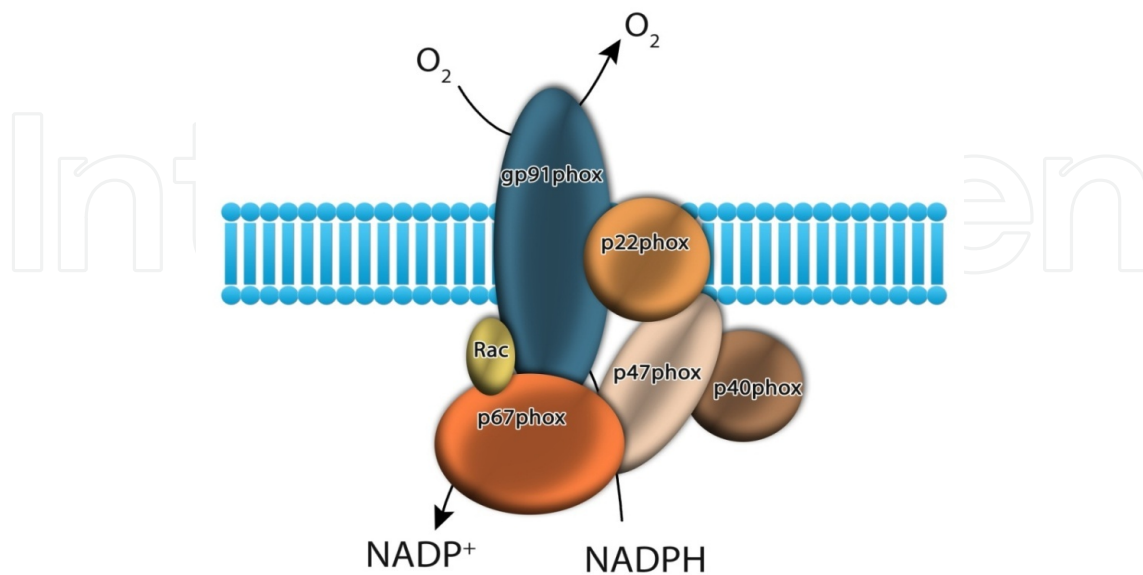






**Figure 8.** Interplay between NADPH oxidase and other sources of ROS

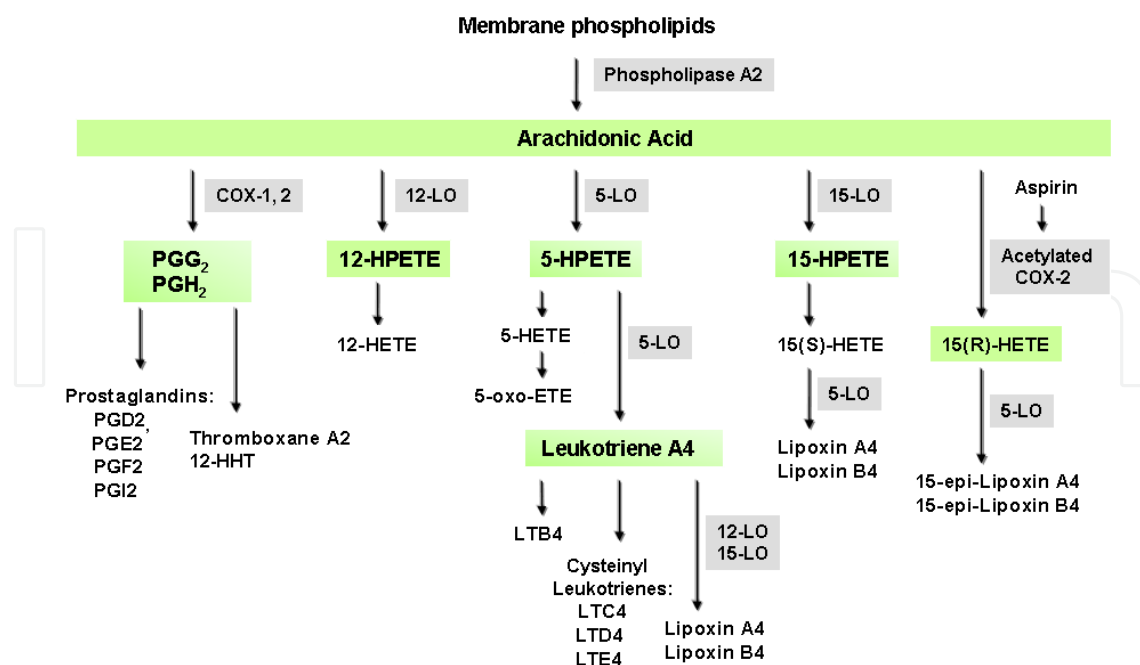
The enzyme is largely derived from leukocytes, such as macrophages and neutrophils and consists of two subunits gp91phox (NOX2) and p22phox. Enzyme complex generates superoxide reducing oxygen via its gp91phox subunit with reduced NADPH as the electron donor. There are lots of NADPH oxidase (NOX) isoform identified in mammalian cells (NOX1, NOX-3, NOX-4, NOX-5, etc.) [53-55]. NADPH oxidase plays a key role in mammalian cells intracellular signaling pathways by generating ROS molecules of NADPH oxidase. ROS signaling system is overwhelming in comparison with other signaling mechanisms [1].



**Figure 9.** Schematic diagram of the structure of the active NADPH oxidase complex

### 3.2.3. Lipoxygenases and ROS

The 5-lipoxygenase (5-LOX) has 78 kDa molecular weight and catalyzes the biosynthesis of potent bioactive eicosanoids, such as leukotriene (LT) and hydroxy eicosatetraenoic acid (HETE). The biosynthesis of LT begins with the metabolism of arachidonic acid by 5-LOX. These soluble dioxygenases incorporate oxygen molecules at position C5 of the fatty acid, yielding 5 (S)-hydroperoxyeicosatetraenoic acid (5-HPETE), which is subsequently metabolized by 5-LOX to generate the unstable epoxide, leukotriene A<sub>4</sub> (LTA<sub>4</sub>). LTA<sub>4</sub> can be converted to LTB<sub>4</sub> via LTA<sub>4</sub> hydrolase, or to LTC<sub>4</sub> via LTC<sub>4</sub> synthase [56]. The most relevant pathophysiological function performed by LTs involves the regulation of inflammatory immune responses. Leukotriene B<sub>4</sub> (LTB<sub>4</sub>) is a potent activator of neutrophil chemotaxis, whereas the cysteinyl leukotrienes (CysLTs) (i.e., LTC<sub>4</sub>, LTD<sub>4</sub>, and LTE<sub>4</sub>) are key mediators of allergic inflammation. LTB<sub>4</sub>, which was the first leukotriene to be isolated, elicits a variety of inflammatory responses, including leukocyte activation, chemotaxis, and degranulation [57]. Over the past few years, it has been reported that LTB<sub>4</sub> treatment of fibroblasts and neutrophils results in ROS generation, and that LTB<sub>4</sub>-induced chemotaxis is mediated by a NADPH oxidase-dependent cascade. These observations suggest that LTB<sub>4</sub>-induced ROS generation occurring via NOX is crucial to cell chemotaxis [58]. LTB<sub>4</sub> has also been previously shown to promote the phosphorylation and translocation of p47phox, thereby stimulating NADPH oxidase. Collectively, these results suggest that the 5-LOX metabolite, LTB<sub>4</sub>, appears to stimulate NOX, thereby generating ROS that mediate a variety of signaling pathways in non-phagocytic cells [59].



**Figure 10.** Generation of various bioactive eicosanoids via the metabolism of arachidonic acid by lipoxygenases and cyclooxygenases

## 4. Conclusion

ROS are inevitable by-products of normal metabolism. ROS are produced in ETC by electron transfer to molecular oxygen. They act as signaling molecules which mediate response in living cells. By the time ROS level increased in cell it causes cellular oxidative damage to DNA, protein and lipid. ROS lead to altered membrane properties. Membrane fluidity and ion transport are impressed. They cause loss of enzyme activities, DNA damage, and inhibition of protein synthesis which result in cell death. Today it is well known that ROS produced in the cells are involved in the redox regulation of signal transduction pathways.

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