

## Oxygen free radicals and pulmonary disease

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Oxygen free radicals are molecules that present unpaired electrons in their outer orbit and can transform other molecules such as proteins, carbohydrates, lipids and deoxyribonucleic acid. Oxygen free radicals are produced in various clinical conditions in which hypoxic microenvironments are generated and reoxygenation follows. Such situations include clinical shock, septicemia, systemic inflammatory response, fulminant hepatitis, organ transplant and respiratory failure. In this review, we discuss the main concepts related to oxygen free radicals: the principal types and their formation, as well as the way in which they affect cellular structures and cause significant tissue damage. We present also the main antioxidants that guard against oxidative stress, including glutathione, glutathione peroxidase, superoxide dismutase, catalase, and N-acetylcysteine. The influence of oxygen free radicals on the principal pulmonary diseases are also discussed, with special emphasis given to oxygen free radicals in cigarette smoke, chronic obstructive pulmonary disease, asthma, sleep apnea syndrome and acute respiratory distress syndrome.

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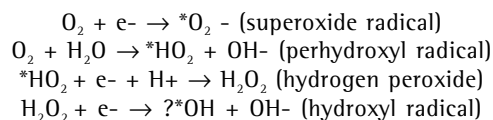
## INTRODUCTION

In recent decades, knowledge regarding oxygen free radicals (OFRs) has generated a lot of interest due to the role that these molecules play in various clinical situations encountered in medical practice. Tissue damage caused by OFRs is seen in various conditions, such as clinical shock, septicemia, systemic inflammatory response, fulminant hepatitis, alcoholic hepatitis, organ transplant, cardiac failure, respiratory failure, etc. What all of these clinical situations have in common is either hypoxic microenvironments followed by reoxygenation or ischemic microenvironments followed by reperfusion. Both conditions facilitate OFR generation.

Oxygen free radicals are atoms or molecules that contain oxygen and present an unpaired electron in their outer orbit. These radicals can react with other molecules by colliding against them, removing their electrons and modifying their molecular structures<sup>(1)</sup>. The main metabolic pathway for oxygen in the organism is related to the complete reduction of oxygen to water, incorporating 4 electrons in the terminal part of the respiratory chain. If oxygen reduction involves a smaller number of electrons along the respiratory chain, intermediate OFRs are produced. The most common OFRs are singlet oxygen, hydroxyl (\*OH), superoxide (\*O<sub>2</sub><sup>-</sup>) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>).

### Production of oxygen free radicals

As previously stated, the complete reduction of molecular oxygen to water is obtained through the reception of 4 electrons in the terminal part of the respiratory chain. However, if oxygen reduction involves a smaller number of electrons, other OFRs can be formed, as shown below:



### The hydroxyl radical

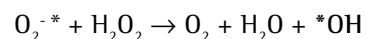
The \*OH radical can be formed by homologous fission of the O-O bond in the H<sub>2</sub>O<sub>2</sub> molecule. The simple mixture of H<sub>2</sub>O<sub>2</sub> with iron salt<sup>(1)</sup> also forms the \*OH radical<sup>(2)</sup>. This reaction was first reported

in 1894 by Fenton. There are two main sources of the \*OH radical in cells:

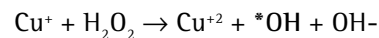
Decomposition of H<sub>2</sub>O<sub>2</sub> by Fenton's reaction:



and H<sub>2</sub>O<sub>2</sub> interaction with the \*O<sub>2</sub> radical by the Haber-Weiss reaction:



The \*OH radical can also be formed when a reduced form of copper comes into contact with H<sub>2</sub>O<sub>2</sub>, as observed in the reaction:



This OFR is the most reactive of all and has the shortest half-life<sup>(1,3)</sup>. It is important to emphasize that the shorter the half-life of an OFR, the more unstable its electronic configuration is and, therefore, the faster it will remove electrons from other molecules. For example, in the case of the \*OH radical, the diffusion capacity prior to reaction is estimated to be only 2 molecular diameters. This OFR reacts significantly with amino acids, phospholipids, deoxyribonucleic acid (DNA), ribonucleic acid and organic acids. There are 3 main reaction types: hydrogen atom subtraction (e.g. methanol); addition of elements to an aromatic ring (e.g. DNA); and electron transfer (e.g. chlorine).

### The superoxide radical

The \*O<sub>2</sub><sup>-</sup> radical is less reactive than the \*OH radical and is formed through the reduction of oxygen by one electron. Under physiological conditions, it is principally generated in the mitochondria, microsomes and peroxisomes<sup>(4)</sup>. Its half-life is longer than that of the \*OH radical, and it can react with molecules for a longer time. Reactions triggered by the \*O<sub>2</sub><sup>-</sup> radical can form \*OH and peroxy radicals. In an acidic environment, these OFRs quickly form H<sub>2</sub>O<sub>2</sub>. In neutral or alkaline environments, \*O<sub>2</sub><sup>-</sup> dismutation is catalyzed by the \*O<sub>2</sub><sup>-</sup> dismutase enzyme (SOD)<sup>(5,6)</sup>. The \*O<sub>2</sub><sup>-</sup> radical presents little molecular reactivity, and its capacity to cause significant damage to cellular structures

is debatable. This OFR can react with nitric oxide, forming peroxyxynitrite, a substance capable of oxidizing and transferring nitrate to, and thereby inhibiting, the amino acids of various pulmonary proteins.

#### Hydrogen peroxide

The  $H_2O_2$  radical is principally formed by the protonated  $*O_2^-$  radical in a low pH environment. Although  $H_2O_2$  is not a real OFR, it can react with redox-active metals such as iron and copper, producing new OFRs. In addition, it has a long half-life and a high capacity to diffuse through hydrophobic cellular membranes (its diffusion capacity is similar to that of water), increasing the toxic effect of reoxygenation.

#### Biological sources of free radicals

The main biological pathway for OFR formation is electron transfer associated with the mitochondrial membranes. It is believed that ubiquinone-cytochrome *b* is the most probable  $*O_2^-$  formation site<sup>(7)</sup>. A great part of the  $*O_2^-$  radicals formed in mitochondria are converted to  $H_2O_2$  by mitochondrial  $*O_2^-$  dismutase, and  $H_2O_2$  molecules may migrate to the cytosol.

Microsomes and nuclear membranes may also participate in the system of electron transportation through the cytochromes P450 and B5, which can produce OFRs. The shift in the position of the cytochrome P450 isoenzyme may also have an influence on the potential for OFR formation through a process that is still unclear. However, it is believed that this phenomenon is related to cytochrome P450 fluctuation through the sum of the high- and low-energy spin states. A shift to the high-energy state might result in increased production of  $*O_2^-$  and  $H_2O_2$  due to greater reduction of the cytochrome P450<sup>(8)</sup>.

Although OFRs are often formed in reoxygenation environments coming from the mitochondrial respiratory chain, they may also be generated by cytoplasmic sources, such as the enzyme xanthine oxidase. Xanthine oxidase catalyzes the reaction of hypoxanthine with oxygen, producing uric acid and  $*O_2^-$  radicals<sup>(9)</sup>. For this reaction to occur, a minimum period of hypoxia, in which there is complete degradation

of ATP to hypoxanthine, as well as conversion of xanthine dehydrogenase to xanthine oxidase, is necessary. Using a model of hepatocyte primary culture, it was demonstrated that the minimum period of hypoxia prior to reoxygenation so that there is significant OFR production is about 2 hours<sup>(10)</sup>. Cells also produce OFR using other sources, including oxidase enzymes (aldehyde oxidase, flavin dehydrogenase, cyclooxygenase, NADPH oxidase, and cytochrome P450 oxidase system), autoxidation of small molecules (catecholamines, flavins, and hydroquinones) and the carrier system for microsomal electrons and nuclear membranes<sup>(11)</sup>.

Due to their short half-life, OFRs are rarely detected in experimental trials. Various methods have been devised in order to overcome this technical difficulty. These methods are based on the detection of stable products formed by the effect of OFRs on specific substrates. Hydroperoxides are stable products formed during the peroxidation of unsaturated lipids such as fatty acids and cholesterol. The most notable methods among those available for hydroperoxide detection are the thiobarbituric acid reactive substances (TBARS) and the chemical method of ferrous oxidation in xylenol orange (FOX). The TBARS method is one of the most widely-used methods for the study of lipid peroxidation and is based on the reaction of malondialdehyde with thiobarbituric acid. It is a simple and sensitive, although not very specific, means of determining lipid peroxidation<sup>(11)</sup>. The FOX method is simple and inexpensive, and it has various technical advantages<sup>(12)</sup>. It is based on the oxidation of  $Fe^{+2}$  (ammonium ferrous sulfate) to  $Fe^{+3}$  caused by hydroperoxide under acidic conditions. In the presence of hydroperoxide, a chemical complex is formed between the iron ion and xylenol orange, producing a bluish-purple color<sup>(13)</sup>. This method is appropriate for studies of biological samples, such those designed to detect hydroperoxides in the membranes of erythrocytes, hepatocytes or other cell types.

#### Systems of defense against oxidative aggression

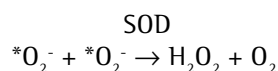
The antioxidant defense system is composed of the suite of substances that neutralize the hazardous effects of OFRs. Enzymes found in the

cytosol, such as SOD, catalase, and glutathione peroxidase, have also been observed in mitochondria, where a great number of OFRs are produced<sup>(14)</sup>.

Recently, Davies<sup>(15)</sup> proposed a comprehensive classification of the antioxidant defense system, divided into primary and secondary defense. Primary defense includes antioxidant complexes such as vitamins (E, A and C), glutathione, uric acid, and antioxidant scavenger enzymes (such as SOD, catalase and the peroxidases). Secondary defense includes lipolytic enzymes, phospholipases, proteolytic enzymes, DNA repair enzymes, endonucleases, exonucleases, and ligases.

The reduced form of the tripeptide glutathione (gamma-glutamyl-cysteinyl-glycine) is the most abundant low-molecular-weight thiol in virtually all mammalian cell systems. The chemical versatility of reduced glutathione, especially its interaction with various oxidant components such as  $^*O_2^-$ ,  $H_2O_2$ , and  $^*OH$ , makes it an efficient reductant. Reduced glutathione is found in high concentrations in bronchoalveolar lavage fluid, conferring protection against oxidative injury to the lungs. The importance of reduced glutathione was confirmed in studies in which its depletion was related to a higher risk of pulmonary disease<sup>(16)</sup>.

The dismutation of  $^*O_2^-$  to  $H_2O_2$  by SOD is frequently included in the primary antioxidant defense since this enzyme is directly involved in the prevention of  $^*O_2^-$  radical accumulation through the following reaction:



The rate of SOD-induced  $^*O_2^-$  dismutation is approximately  $10^4$  times greater than that of chemical dismutation<sup>(17)</sup>. There are 3 distinct types of  $^*O_2^-$  dismutase, classified based on the metal they contain: Cu/Zn-SOD (cytosol), Mn-SOD (mitochondria) and Fe-SOD. Extracellular SOD is plentiful in pulmonary tissue and protects the lungs against oxidative stress. However, its role in asthma and other airway diseases is still unclear<sup>(18)</sup>. In animal models, extracellular SOD seems to play an important role in reducing OFR-induced pulmonary injury after the administration of bleomycin<sup>(19)</sup>.

The catalase enzyme is considered the major component of the primary antioxidant defense and, together with glutathione peroxidase, is involved in the catalysis of  $H_2O_2$  decomposition into water. If the level of  $H_2O_2$  is low, organic peroxides are preferably eliminated by glutathione peroxidase. However, if there is high  $H_2O_2$  concentration, the action of catalase is predominant. Catalase may be protective against some tumors, such as lung cancer tumors. In a study comprising 24 patients diagnosed with lung cancer, catalase activity was significantly lower in tumor tissue than in normal lung tissue<sup>(20)</sup>.

N-acetylcysteine is a mucolytic drug and has antioxidant properties since it is a precursor of reduced glutathione. The use of this substance in 1219 patients hospitalized with decompensated chronic obstructive pulmonary disease (COPD) reduced, in a dose-dependent fashion, the risk of readmission by 30%<sup>(21)</sup>. In a meta-analysis, it was concluded that the prolonged use of N-acetylcysteine reduced the number of acute exacerbations in patients with chronic bronchitis<sup>(22)</sup>. The long-term use of N-acetylcysteine also helped reduce acute exacerbations in patients with moderate to severe COPD<sup>(23)</sup>. In patients with COPD, the long-term use of N-acetylcysteine decreased levels of exhaled  $H_2O_2$ , suggesting that the beneficial effect of this substance is related to this mechanism<sup>(24)</sup>.

## Cellular damage caused by oxygen free radicals

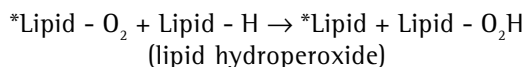
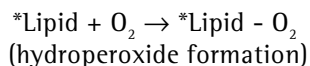
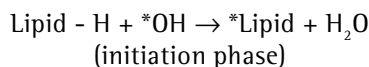
### Free radical reaction with proteins

The OFR oxidation of amino acids induces physical changes in the proteins they compose. These changes fall into three categories: fragmentation, aggregation, and susceptibility to proteolytic digestion<sup>(25)</sup>. The phenomenon of OFR-related fragmentation has been documented for albumin and collagen<sup>(26,27)</sup>. Proteins are selectively fragmented in proline residues ( $^*OH$  radicals), as well as in histidine and arginine amino acids, which are closely associated with transition metals. The  $^*OH$  radicals may be those most responsible for protein aggregation because of their capacity to form cross-links between these proteins. Proteolytic degradation results from crude alterations in

protein conformation, possibly related to the OFR effect.

#### Free radical reaction with lipids

*In vitro* studies have shown that peroxidation of polyunsaturated fatty acids (lipid peroxidation) usually involves three, operationally defined, phases: initiation, propagation, and termination<sup>(28)</sup>. The initiation phase occurs with the formation of a diene conjugate resulting from the removal of a hydrogen atom by a sufficiently reactive OFR<sup>(2)</sup>. The propagation phase involves interaction between molecular oxygen and carbon, forming the hydroperoxide radical that removes hydrogen atoms from other lipid molecules, resulting in lipid hydroperoxide<sup>(2)</sup>. With the aid of catalytic metals, hydroperoxide decomposition forms alcoxyl and peroxy radicals that can start a chain reaction, propagating lipid peroxidation. The following sequence of reactions illustrates this phenomenon:



Lipid peroxidation is the major source of cytotoxic products, such as aldehydes, that are produced by hydroperoxide decomposition<sup>(29)</sup>. The principal fatty acids that undergo lipid peroxidation at the cellular level are, among others, the linoleic, arachidonic, and docosahexaenoic acids<sup>(1)</sup>.

#### Carbohydrate peroxidation

Sagone Jr *et al.*<sup>(30)</sup> showed that glucose oxidation may be both a means of eliminating hydroperoxide radicals and a source of OFRs. Wolff *et al.*<sup>(31)</sup> demonstrated that simple monosaccharides are rapidly submitted to autoxidation under physiological conditions, forming dicarbonyl and  $\text{H}_2\text{O}_2$ . Oxidized glucose can react with proteins in a process called glycosylation or glycation.

#### Genome modification

Approximately 20 types of OFR-induced oxidative changes to DNA have been observed. The estimated damage level ranges from 8 to 83 residues per  $10^6$  deoxyguanosine residues, increasing with age and affecting the liver, kidney, and spleen but not the brain<sup>(32)</sup>. Mitochondrial DNA damage is noteworthy since mitochondria are the major OFR source and mitochondrial DNA is exposed to high levels of free radicals. Therefore, mitochondrial DNA seems to be the preferential target of various carcinogenic xenobiotic chemicals<sup>(33,34)</sup>. Damage to DNA induced by  $\cdot\text{OH}$  radicals includes base changes and molecular cleavage. Of the five DNA components, thymine and cytosine are the bases most susceptible to attack by  $\cdot\text{OH}$  radicals, followed by adenine, guanine and deoxyribose sugar<sup>(35)</sup>.

#### Pulmonary diseases and oxidative stress

##### Sources of free radicals in the lungs

Various cells of the lung parenchyma, such as endothelial cells, type-2 alveolar cells, Clara cells, airway ciliated cells and alveolar macrophages, are capable of producing OFRs<sup>(36)</sup>. The systems that generate OFRs in the lungs are similar to those seen in other tissues.

##### Relationship between free radicals and lung damage

In most cases, “ischemia/reperfusion” and “anoxia/reoxygenation” are corresponding terms. However, in the pulmonary tissue, there is a difference between these two concepts since oxygen is present in the alveoli during lung ischemia. Under this condition, alveolar oxygen helps maintain aerobic metabolism, postponing hypoxia<sup>(37)</sup>. In addition, in contrast to other tissues, the lungs come into contact with oxygen in two different ways: perfusion and ventilation. Hypoxia or anoxia results in a dramatic decrease in the levels of adenosine triphosphate (ATP) and more intense ATP degradation, resulting in increased hypoxanthine production. When oxygen is reintroduced into the environment through reperfusion or ventilation, the  $\cdot\text{O}_2^-$  radical is formed by the effect of xanthine oxidase on hypoxanthine. Xanthine oxidase inhibitors, such as allopurinol, can block this phenomenon<sup>(38)</sup>. The absence of blood flow in the lungs may cause lipid

peroxidation and oxidative damage due to the presence of oxygen<sup>(39)</sup>. This type of damage is not related to ATP depletion and therefore cannot be blocked by xanthine oxidase inhibitors<sup>(38)</sup>. The endothelium seems to be one of the major oxidant sources in nonhypoxic lung ischemia caused by the activation of nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase and of the NF- $\kappa$ B factor, as well as by calcium/calmodulin-dependent nitric oxide synthase<sup>(40,41)</sup>. Nitric oxide is a simple diatomic gas and generates the two most important reactive nitrogen substances found in biological systems: peroxyxynitrite and peroxyxynitrous acid.

Other cells presenting high NADPH-oxidase activity, such as macrophages and neutrophils, can also contribute to oxidative damage in the lungs.

#### Cigarette products

The significance of cigarette smoking in OFR-induced pulmonary lesions has been proven in various experiments. Compared to nonsmokers, chronic smokers have higher plasma levels of lipid peroxidation products, measured through the thiobarbituric acid (TBA)-malondialdehyde method<sup>(42)</sup>. In addition, there is a decrease in antioxidants in the distal airways of smokers, as well as a decrease in vitamin E in the bronchoalveolar lavage fluid, when compared to nonsmokers<sup>(42)</sup>. Due to the lipophilic properties of tocopherol, vitamin E is the major free radical scavenger in lipid environments and, in the intracellular environment, is related to lipid-rich membranes such as that of the endoplasmic reticulum. There is also evidence that the leukocytes of smokers release more OFRs<sup>(43)</sup>.

Cigarette smoke induces high OFR levels in the human airways<sup>(20)</sup> and may cause inflammation and higher protease release due to higher OFR production. This phenomenon is counterbalanced by antiproteases that are designed to prevent damage to the lung parenchyma. Inadequate antiprotease production might result in failure to neutralize proteases activated by smoking, leading to the onset of COPD<sup>(44)</sup>. In an interesting animal model study, it was shown that cigarette smoke caused vitamin A depletion in rats, and that this depletion was correlated with the development of

emphysema. Benzopyrene, a component of cigarette smoke, is responsible for vitamin A depletion<sup>(45)</sup>. Carotenoids protect lipids against peroxidation caused by free radicals and other reactive oxygen species, especially singlet oxygen.

#### Chronic obstructive pulmonary disease

Production of  $^*O_2^-$  radicals by the neutrophils of patients with COPD and acute exacerbation seems to be significantly higher than in healthy individuals of the same age. Plasma levels of lipid peroxidation products, measured using the TBARS technique, are higher in COPD patients than in normal individuals and are even higher in patients presenting acute exacerbation of the disease<sup>(44)</sup>.

Levels of isoprostane-F2 (a prostaglandin isomer formed through lipid peroxidation) in urine are significantly higher in patients with COPD (mean of 84 pmol/mmol of creatinine) than in control patients (mean of 35.5 pmol/mmol of creatinine)<sup>(46)</sup>. These results are also indicative of the role of oxidative stress in the physiopathology of COPD.

Patients with acute COPD exacerbations present increased serum levels of malondialdehyde (a product of lipid peroxidation), which return to normal levels after treatment<sup>(47)</sup>. In view of this, serum levels of malondialdehyde were significantly higher in patients with COPD, with or without acute exacerbations, than in control individuals<sup>(47)</sup>. In addition, erythrocyte glutathione and serum levels of vitamin C (ascorbic acid) were lower in patients with acute exacerbations of COPD than in control patients<sup>(48)</sup>. Vitamin C is hydrophilic and reacts with  $^*O_2^-$ ,  $H_2O_2$  and various lipid hydroperoxides. In addition, vitamin C can restore the antioxidant properties of oxidized vitamin E.

#### Asthma

Asthma is a disease characterized by chronic airway inflammation. The production of OFRs by activated inflammatory cells causes various physiopathological changes associated with asthma. The activities of antioxidant enzymes and their relationship with asthma are as yet unclear. In a study involving patients with mild asthma,

SOD activity was significantly lower in those patients than in controls<sup>(49)</sup>. In another study, extracellular glutathione peroxidase levels were higher in the airways of asthmatic patients than in those of controls<sup>(50)</sup>.

Plasma levels of lipid peroxidation products, as measured using TBA-malondialdehyde technique, were significantly higher in chronic asthmatic patients than in normal individuals<sup>(41)</sup>. In asthma, an increase in the oxidant load may result in the release of reactive oxygen intermediates<sup>(41)</sup>, as well as of nitric oxide. The  $^*O_2^-$  anions and nitric oxide quickly interact to form peroxynitrite (ONOO<sup>-</sup>), which has considerable oxidative capacity.

#### Obstructive sleep apnea

There is ample evidence that there is increased release of OFR by circulating neutrophils during obstructive sleep apnea since OFRs can reduce nitric oxide, which is an endothelial vasodilator (patients with obstructive sleep apnea have decreased serum levels of nitric oxide derivatives)<sup>(51)</sup>, and lipid peroxidation increases<sup>(52)</sup>. In addition, OFRs cause an increase in platelet aggregation, a phenomenon that also occurs in obstructive sleep apnea, and can increase the expression of various endothelial genes, such as those responsible for the synthesis of adhesion molecules, endothelin, and vascular endothelial growth factor. Therefore, patients suffering from obstructive sleep apnea present increased expression of vascular cell adhesion molecule, intercellular adhesion molecule and E-selectin<sup>(53)</sup>, as well as greater induction of vascular endothelial growth factor<sup>(54)</sup>.

#### Acute respiratory distress syndrome

Acute respiratory distress syndrome is characterized by diffuse inflammation of the lung parenchyma. Patients with this syndrome suffer from oxidative stress from two main sources: activated neutrophils and high oxygen levels employed during ventilatory therapy. The involvement of inflammatory mediators in acute respiratory distress syndrome has been investigated in depth, and tissue damage mediated by oxidative

agents seems to be important in the pathogenesis of the disease. In response to various inflammatory stimuli, pulmonary endothelial cells, alveolar cells, airway epithelial cells and activated alveolar macrophages produce nitric oxide and  $^*O_2^-$ , which react with one another forming ONOO<sup>-</sup>. This radical can oxidize key amino acids in various proteins in the lungs – surfactant protein A, for example – inhibiting their functions<sup>(55)</sup>. It has been suggested in various experimental studies that both reactive nitrogen radicals and OFRs are involved in the pathogenesis of acute respiratory distress syndrome<sup>(56)</sup>. There is evidence that the antioxidant system is defective in patients with this syndrome. In one study, plasma levels of alpha-tocopherol, vitamin C, beta-carotene, and selenium were reduced in patients with this disease<sup>(57)</sup>.

## CONCLUSIONS

The OFRs are molecules that contain oxygen and present unpaired electrons in their outer orbit. This characteristic makes OFRs a source of problems for cells and tissues, since they are able to react with and modify the molecular structures of lipids, carbohydrates, proteins and DNA. The cellular metabolism itself can produce OFRs via the mitochondria (the principal OFR producer) and cytoplasm, but the production rate dramatically increases if tissues are exposed to hypoxic microenvironments followed by reoxygenation (or to ischemia followed by reperfusion). There is a minimum hypoxia/ischemia period prior to reoxygenation/reperfusion necessary for the production of a great number of OFRs. Since lungs come into contact with oxygen in two different ways (perfusion and ventilation) they frequently become OFR targets, and various pulmonary diseases seem to be influenced by these molecules. There is evidence that OFRs are related to tissue damage in chronic tobacco smoking, COPD, asthma, obstructive sleep apnea, acute respiratory distress syndrome, and other conditions. This knowledge has evolved in parallel with the study of antioxidant agents able to neutralize the effects of OFRs. In the future, the use of OFR neutralizing agents may be included in the therapeutic arsenal against the principal pulmonary diseases.

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