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Mechanisms of Pulmonary Oxygen Toxicity

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The oxygen molecule has unique characteristics which help explain both its important role in normal oxidative metabolism and its toxic reactions. The oxygen molecule contains 16 electrons, the first eight of which are paired in 1S and 2S orbitals. The last eight electrons in the oxygen molecule are located in π orbitals. Six of these exist as pairs (with opposite spins) in three bonding orbitals. The final two electrons are located in two different anti-bonding orbitals of equal energy levels. The lowest energy state for the oxygen molecule is for these last two electrons to be in separate orbitals and for both to have the same spin. This means that the oxygen atom contains two unpaired electrons in its outer shell, each with the same spin. The oxygen molecule can accept four additional electrons to complete the antibonding π orbitals. The oxygen molecule functions as the primary electron sink for cellular metabolism. It thus plays the key role of accepting electrons from a wide variety of metabolic reactions and, in the process, releasing the free energy necessary to carry out energy dependent cell processes.

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The two unpaired electrons in the outer orbital of the oxygen molecule give it unique reaction characteristics. Most organic molecules found in animal cells have only paired electrons. An electron pair cannot readily react with oxygen because paired electrons have opposite spins. This creates a spin resistance against one of the two electrons in a pair entering the available orbitals on an oxygen molecule. For an electron pair to enter the oxygen molecule, one of the electrons would have to change spin. Electrons change their spin approximately 100 million times per second; however, this is only one ten millionth of the time available during a molecular collision reaction. Thus, a collision reaction of an oxygen molecule with most other organic molecules is a spin forbidden process. It will not occur. Because of this, the oxygen molecule can move across the alveolar capillary membranes, bind to hemoglobin, be transported to peripheral tissues and then diffuse into the interior of another cell without initiating uncontrolled reactions. Inside the cell oxygen can be bound by enzymes and then electrons transported onto the oxygen molecule. Since this does not involve a collision reaction there is sufficient time available for electrons to change their spins and enter the available orbitals on the oxygen molecule.

This unique characteristic of oxygen that

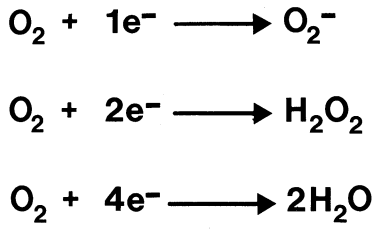

Fig. 1. Products produced during the biological reduction of oxygen.

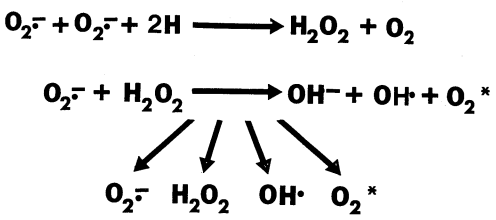

Fig. 2. Reactions of partially reduced species of oxygen to produce additional potentially toxic species.

prevents it from entering into uncontrolled collision reactions allows it to be the key to life in an aerobic atmosphere, but it also is its Achilles heel. It is possible for the oxygen molecule to escape from some oxidases before it is fully reduced. If only one electron is transported to the oxygen molecule the product is superoxide (O_2^-) which has only one unpaired electron **(Fig. 1)**. If two electrons are transported onto oxygen the product is hydrogen peroxide (H_2O_2); while if four electrons are transported to oxygen the product is water. This latter reaction is the preferred one since it produces a harmless waste product and releases the maximum amount of free energy. A four electron reaction with oxygen occurs in mitochondria at the terminal cytochrome, a, a_3. Greather than 95% of oxygen consumption goes through this pathway. Unfortunately, most of the other oxidases in mammalian cells are not capable of transferring four electrons to oxygen and produce either superoxide or hydrogen peroxide as their product. These partially reduced species of oxygen can combine with each other to form additional toxic species. Two superoxide molecules can react together to produce hydrogen peroxide; superoxide plus hydrogen peroxide can react together to form the hydroxyl radical (OH•) and singlet oxygen (O_2*). Thus, once superoxide is formed, further reaction can lead to the production of four, potentially toxic, partially reduced species of oxygen: superoxide, hydrogen peroxide, the hydroxyl radical and singlet oxygen **(Fig. 2)**. The hydroxyl radical is one of the most potent free radicals known. It is equivalent to three electron reduced oxygen, and is one of the primary free radicals produced by ionizing radiation.

All of the partially reduced species of oxygen have few barriers against uncontrolled reactions and can initiate a wide varieties of different destructive reactions. For example, the orderly structure of the normal lipid membrane can be markedly disrupted by oxidative injury. After exposure to free radical mediated injury a wide variety of toxic reactions can occur which are illustrated in **Fig. 3.** These include protein strand scission, protein-protein crosslinking, disulfide crosslinking, lipid-to-protein crosslinks, fatty acid oxidation, lipid-to-lipid crosslinks and amino acid oxidation (1). Hydroxyl radical can also react with both the primary bases that make up DNA and with the ribose-phosphate backbone of DNA causing DNA strand scission (1,2).

The toxic reactions of partially reduced species of oxygen with lipids, proteins and molecules such as DNA are normally balanced by quenching reactions with antioxidants which scavenge the products of the oxidative reactions **(Fig. 4)**. In addition, specific enzymatic defenses exist within the

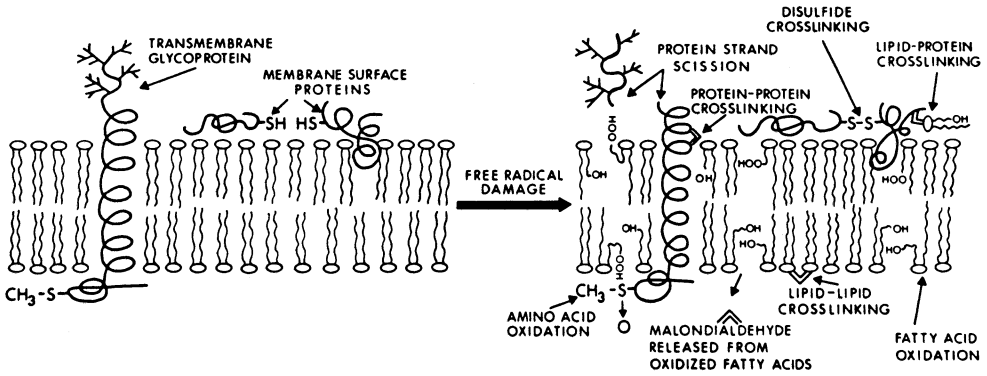

Fig. 3. Free radical damage to membranes. Free radicals can initiate lipid peroxidation which leads to short chain fatty acyl derivatives and to the byproduct malendialdehyde. Free radicals can also catalyze amino acid oxidation, protein-protein crosslinking and protein strand scission (1). (Reproduced with the permission of Laboratory Investigation)

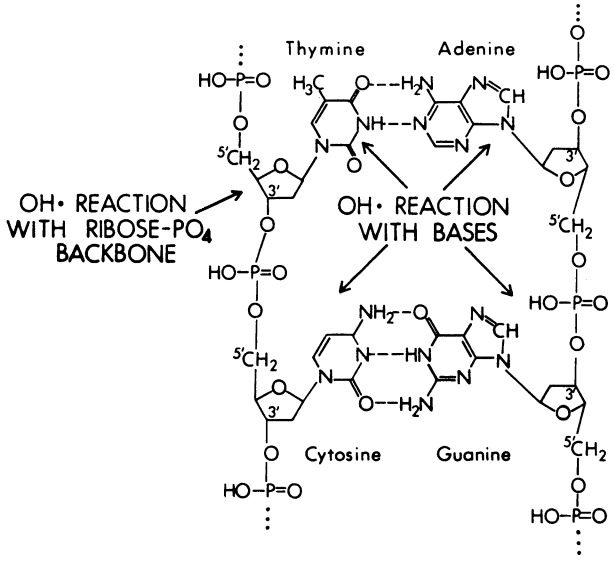

Fig. 4.
Reaction of DNA with hydroxyl radical. Hydroxyl radical can react with and modify DNA bases. DNA strand scission also results from OH· reactions with the ribose phosphate backbone (1). (Reproduced with permission of Laboratory Investigation)

cell which scavenge the partially reduced species of oxygen. Superoxide dismutase (SOD) converts superoxide to hydrogen peroxide while catalase and peroxidases reduce hydrogen peroxide to water.

One of the fundamental postulated mechanisms for hyperoxic mediated tissue injury is that as a result of oxidative metabolism superoxide and hydrogen peroxide are produced. The majority of these are scavenged by cell defense systems while a few escape and recombine to form additional toxic species, including the hydroxyl radical and singlet oxygen. Each of these partially reduced species of oxygen can initiate tissue injury which is balanced by normal tissue repair processes. In the presence of high oxygen tensions, it is postulated that the rate of oxygen radical production from metabolic processes increases, leading to increased numbers of the free radicals escaping the defense systems and causing greater levels of tissue injury which cannot be adequately balanced by repair processes.

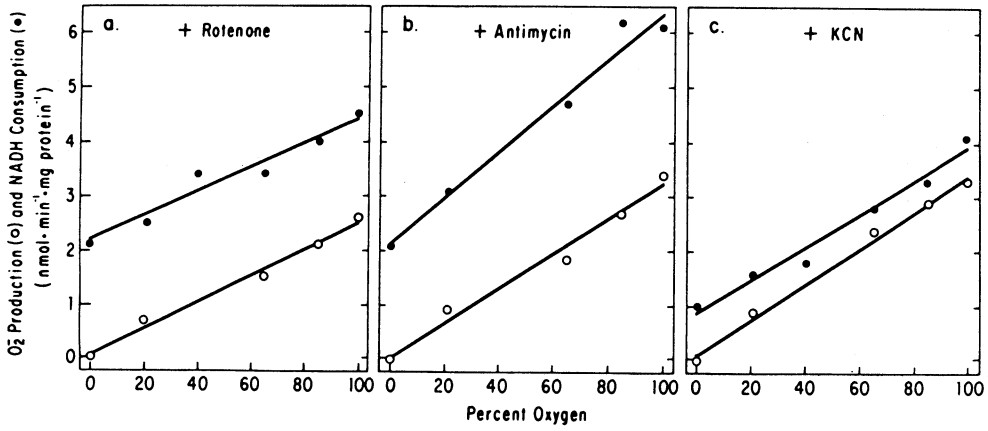

Fig. 5. Superoxide production (open circles) and NADH oxidation (solid circles) by porcine lung submitochondrial particles incubated with 0.1 mM NADH and either 2 μM rotenone (a), 2 μM antimycin (b), or 1.0 mM CN^- (c). Superoxide formation was measured in the presence of 1 mM epinephrine. 0.2 to 0.4 mg/ml submitochondrial particle protein was used (3). (Reproduced with the permission of Archives of Biochemistry and Biophysics)

Intracellular sites of oxygen radical production

The potential intracellular sources of superoxide anion production include mitochondria, endoplasmic reticulum, membranes such as cytoplasmic and nuclear membranes and organelles such as lysosomes and peroxisomes. Data is available on the rates of production of partially reduced species of oxygen by mitochondria, endoplasmic reticulum and nuclear membranes.

Submitochondrial particles, prepared by isolating mitochondria and sonicating them, can be used to evaluate mitochondrial superoxide production since the endogenous antioxidant enzymes can be washed out and the rate of oxygen radical production by the electron transport chain directly assayed. **Fig. 5** shows the rates of superoxide production by lung submitochondrial particles in the presence of three agents that block at different sites along the mitochondrial electron transport chain (3). Note that the rate of superoxide production increases with increasing oxygen tension and that the rate of superoxide production is slightly higher with antimycin and cyanide than it is with rotenone. Two sites on the mitochondrial electron transport chain have been shown to produce superoxide **(Fig. 6)**. One is the NADH dehydrogenase complex and other is the ubiquinone cytochrome b region. Because rotenone blocks between these two sites, it allows only one site to produce superoxide and gives a lower net rate of superoxide production than that which occurs when either antimycin or cyanide are used as the blocking agent. In lung tissue superoxide generation by the NADH dehydrogenase complex is approximately 1.5-fold greater than superoxide production by the ubiquinone-cytochrome b segment (3).

Intact mitochondria contain superoxide dismutase which scavenges superoxide and produces hydrogen peroxide. Even under hyperoxic conditions no superoxide can be found escaping from isolated, intact mitochondria. However, substantial amounts of hydrogen peroxide do escape from intact mitochondria (4). **Fig. 7** shows the rate of

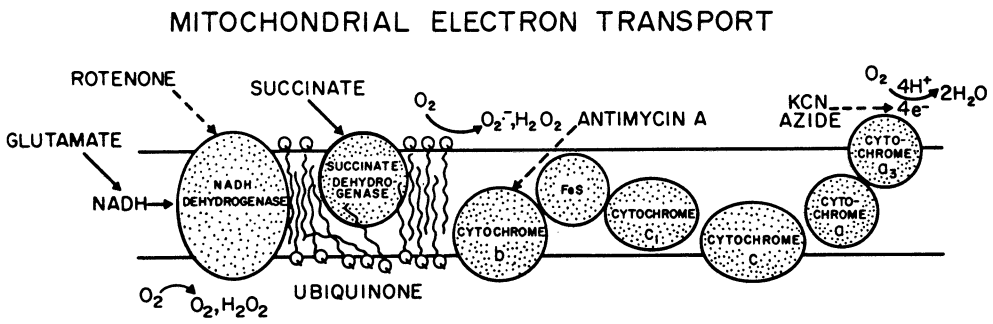

Fig. 6. Mitochondrial free radical generation. Free radicals are produced on the electron transport chain which is located on the inner mitochondrial membrane. Mitochondrial sources of O_2^- have been studied using various electron transport inhibitors (rotenone, antimycin A, KCN, and azide, dashed arrows) and substrates (NADH-linked substrates and succinate, solid arrows). NADH dehydrogenase and the ubiquinone-cytochrome b region have been shown to reduce oxygen to O_2^-(1). (Reproduced with permission of Laboratory Investigation)

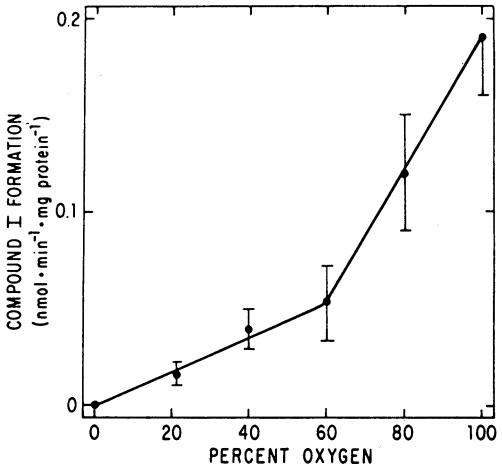

Fig. 7. Effects of oxygen concentration on succinate supported H_2O_2 release by porcine lung mitochondria. Hydrogen peroxide production was measured by monitoring the formation of compound I between H_2O_2 and added horseradish peroxidase. The abrupt increase observed above 60% oxygen was characteristic for each independent experiment (4). (Reproduced with permission of Archives of Biochemistry and Biophysics)

hydrogen peroxide release from isolated intact mitochondria. The rate of hydrogen peroxide release increases with increasing oxygen tension. Note that the overall rate of H_2O_2 release increases significantly above 60% oxygen. This correlates with the well documented greater toxicity of oxygen when the concentration is over 60% (1ATA) and the steep dose-response curve above this point. In the experiments shown in Figure 7 succinate was used as a substrate. The response was typical of findings for other substrates used to support mitochondrial oxidation such as malate+malonate+glutamate (4).

Oxygen radical production by endoplasmic reticulum and nuclear membranes has also been studied and correlated with the oxygen tension. Two sites of oxygen radical production have been identified on endoplasmic reticulum. These include the cytochrome b_5 and cytochrome P450 regions. Cytocrome P450 uses NADPH as a substrate while cyto-

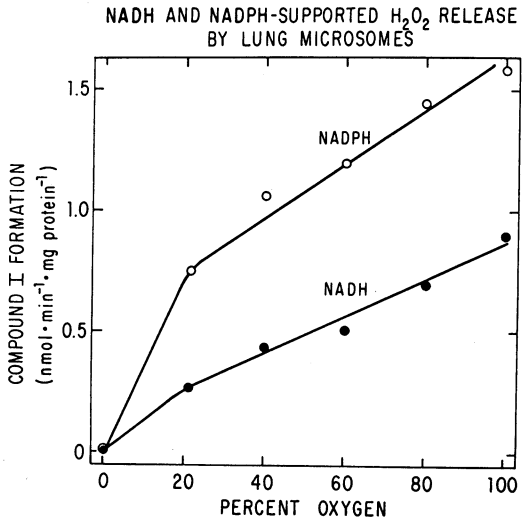

Fig. 8. Effect of oxygen concentration on the NADPH (open circles) and NADH (solid circles) supported H_2O_2 production by porcine lung microsomes. Hydrogen peroxide production was measured by monitoring the formation of compound I between H_2O_2 and added horseradish peroxidase (4). (Reproduced with permission of Archives of Biochemistry and Biophysics)

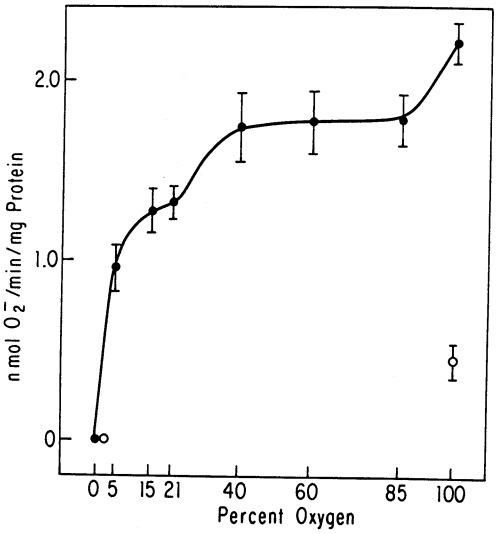

Fig. 9. Superoxide generation by porcine lung nuclei. The solid circles indicate NADH-dependent superoxide generation and open circles indicate NADPH-dependent superoxide generation (5). (Reproduced with permission of Biochimica et Biophysica Acta)

chrome b_5 uses NADH as its primary substrate. When endoplasmic reticulum is isolated from a cell the membranes reform into small vesicles which are termed microsomes. These microsomes can be shown to release significant amounts of hydrogen peroxide, and the rate of this hydrogen peroxide release increases as a function of oxygen tension **(Fig. 8)**. When NADPH is used as a substrate for porcine lung microsomes the rate of hydrogen peroxide production is greater than it is when NADH is used as the substrate (4). This suggests that both cytochrome P450 and cytochrome b_5 produce oxygen radicals but that cytochrome P450 is the dominant source. Nuclear membranes carry out similar reactions to those of endoplasmic reticulum and the rate of superoxide production by nuclear membranes also increases with the oxygen tension **(Fig. 9)**. A unique characteristic of nuclear membranes from lung was that NADH gave a higher rate of hydrogen peroxide production than did NADPH (5). This suggests that on nuclear membranes cytochrome b_5 may be more important than P450 in terms of production of oxygen radicals.

Immunocytochemical Localization of the Superoxide Dismutases

To determine the distribution of antioxidant enzymes and how this compares with the sites where oxygen radicals are produced immunocytochemical studies have been carried out to localize these antioxidant enzymes. There are two superoxide dismutases in mammalian cells. One contains Cu and Zn, has a

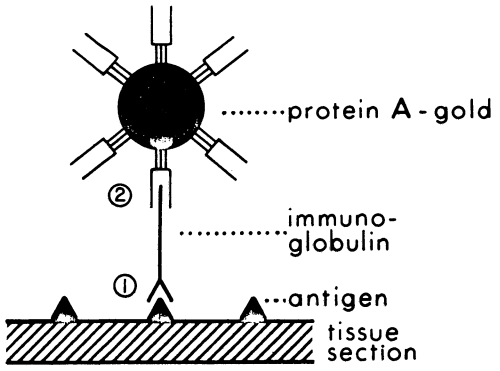

Fig.10. Schematic of immunocytochemical labelling using protein A gold.

molecular weight of 32,000 and is found in the cell cytosol. The other contains Mn, has a molecular weight of 80,000 and is found in mitochondria. Both the CuZu and the manganese forms of superoxide dismutase have been purified from rat liver and antisera were raised in rabbits. Immunocytochemical labelling was done by a sandwich technique in which the primary anti-superoxide dismutase immunoglobulin was first reacted with cryoultrathin sections of tissue (6). This was followed by reaction with protein-A gold **(Fig. 10)**. The colloidal gold particles are electron dense and are coated with protein-A which is a staphococcal protein having a high affinity for the Fc fragment of rabbit immunoglobulin. **Fig. 11** illustrates the localization of the CuZu superoxide dismutase. Each colloidal gold granule represents one antigen site where the enzyme has been identified. Note that the staining is distributed almost uniformly throughout both the nucleus and the cytoplasm. The mitochondria are not stained and the stain is excluded from the cysternae of the endoplasmic reticulum. This staining is characteristic of a soluble phase enzyme. The protein appears to be able to move into the nucleus through the nuclear pores. Its localization is appropriate to protect DNA from oxygen radical injury and to protect against superoxide produced on the endoplasmic reticulum since it is on the appropriate side of the endoplasmic reticulum membrane. **Fig. 12** shows the localization of the man-

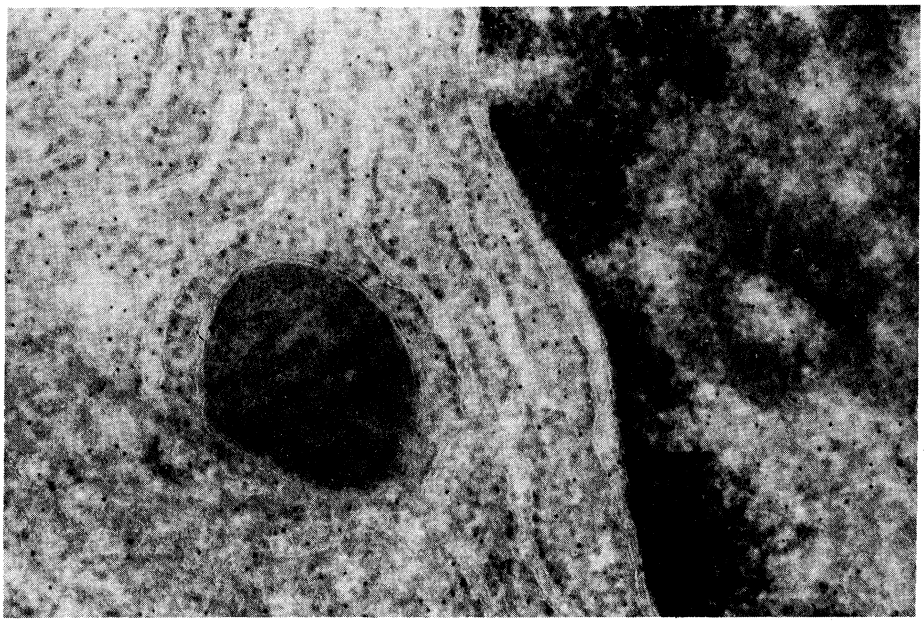

Fig. 11. Cryoultrathin section of rat liver stained with protein A gold for the CuZn superoxide dismutase.

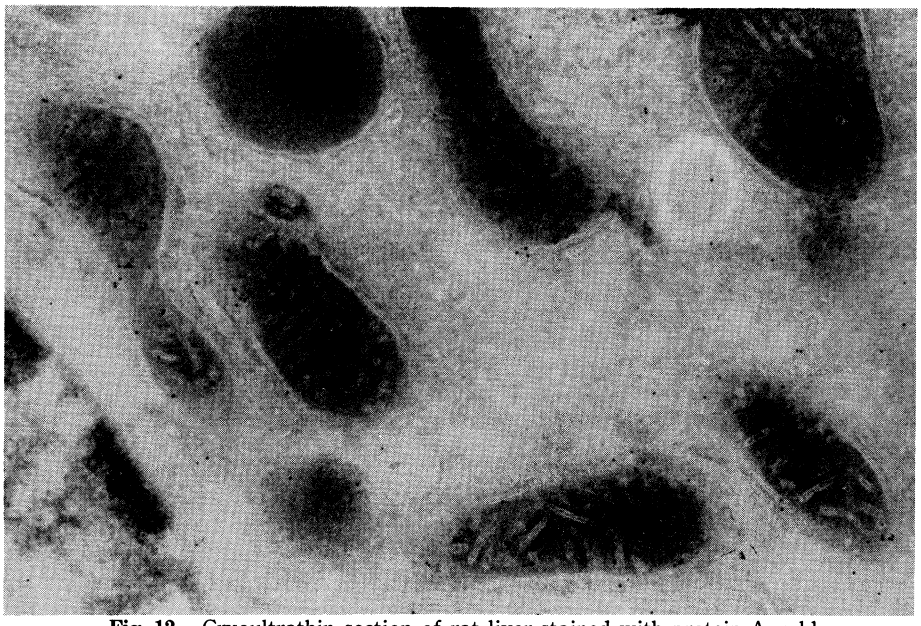

Fig. 12. Cryoultrathin section of rat liver stained with protein A gold for the Mn superoxide dismutase.

ganese superoxide dismutase. It is almost exclusively limited to the mitochondria. The cytoplasm is not stained except for an occasional gold particle found over endoplasmic reticulum. Since most mitochondrial proteins are known to be synthesized on the endoplasmic reticulum this staining may be specific for fragments of the manganese superoxide dismutase at its site of synthesis.

Major Histologic Stages in the Development of Oxygen Toxicity.

The major stages in the progression of pulmonary oxygen toxicity are shown on **Fig. 13** for both lethal and sublethal exposures to hyperoxia (7). The initial reaction in both cases is an initiation of cell injury by the production of increased numbers of partially reduced species of oxygen. During the early phases of injury, cell metabolic and functional changes may occur before there is histologic evidence of injury. An inflammatory phase follows which is rapidly succeeded by a destructive phase. If the injury is sufficiently intense, the animal dies after manifesting symptoms for only a short time. If the injury is less intense, the onset of the inflammatory and the destructive phases are delayed. A proliferative phase then starts which may prevent the death of the animal. In spite of continued exposure to hyperoxia, the magnitude of the inflammatory reaction subsides. If these injuries are continued long enough, a fibrotic phase will occur which will result in permanent derangement of lung structure and function.

The histologic events which characterize these stages have been quantified using morphometric techniques (8). During exposure to a lethal dose of hyperoxia, the earliest histologic reaction is an increase in the number of platelets in the lung microvasculature (9). This is followed by a rapid influx of neutrophils which have been postulated by some investigators to be an important component in the augmentation phase of the injury (9). The inflammatory phase is rapidly followed by a destructive phase which, in small laboratory

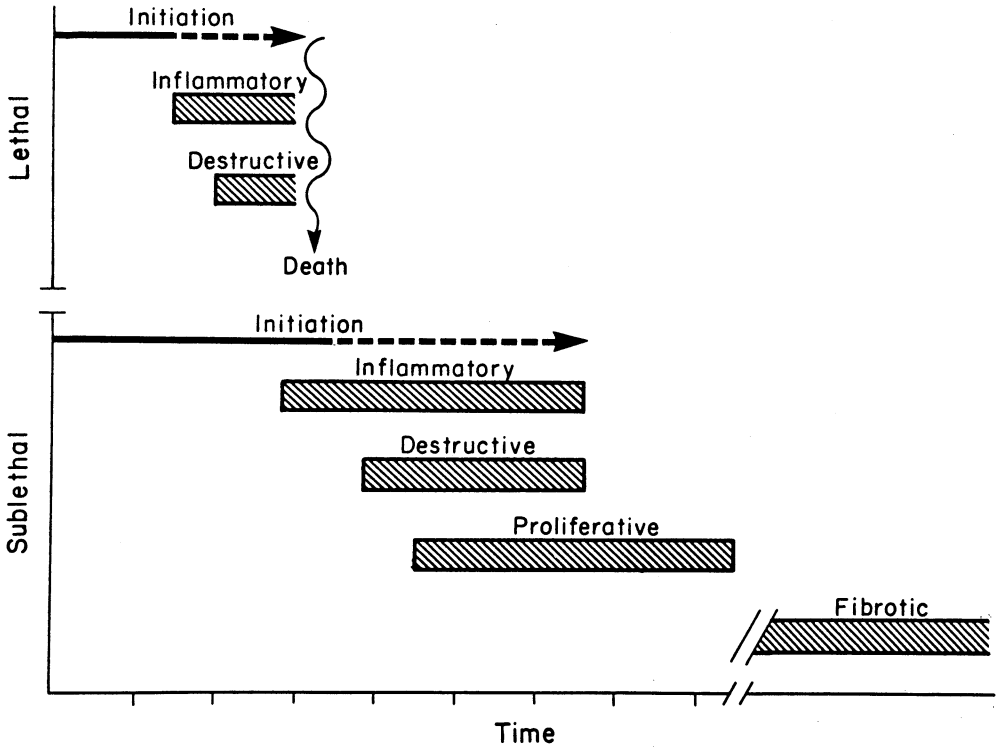

Fig. 13. Relative time course of each major phase of lung injury caused by lethal and sublethal exposures to hyperoxia. The metabolic events associated with initiation of the injury do not cause morphologic changes during the earliest phase (7). (Reproduced with permission of Annual Review of Physiology)

animals, predominantly involves damage to the capillary endothelium. In rats, almost half of the capillary endothelial cells are destroyed over a few of hours once this phase starts. This leads to the death of the animal after 60 to 72 hours of exposure to 100% O_2. If the exposure to hyperoxia is at a sublethal level, the onset of the inflammatory stage is similar, but delayed. The destructive phase again occurs, but before the extent of injury is sufficient to be lethal, a proliferative phase can be identified. This is associated with proliferation of both epithelial cells and interstitial cells. Destruction of the capillary endothelium halts. The onset of the late fibrotic phase is characterized by an increase in inflammatory cells, fibroblasts and collagen in the alveolar septae.

The postulated mechanisms in the early processes of oxygen toxicity are further illustrated in **Fig. 14.** The initiation steps of oxygen toxicity are thought to be intracellular and to be caused by enhanced rates of production of oxygen radicals from subcellular sites such as mitochondria, endoplasmic reticulum and nuclear membranes. This leads to alterations in cell metabolism as well as alteration of lipid membranes on the cell surfaces. Activation of macrophages and release of mediators from macrophages as well as direct release of mediators by the endothelium occur. These mediators include eicosanoids, fatty acid oxidation products and peptides. The inflammatory stage begins

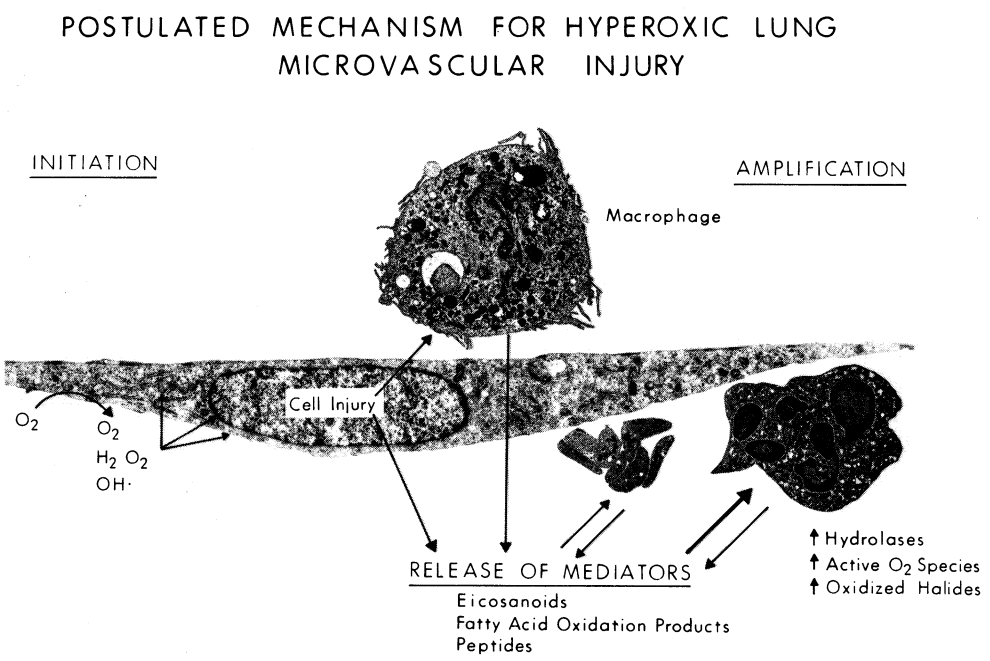

Fig. 14. Early events in the pathogenesis of pulmonary oxygen toxicity. The earliest phase is associated with production of partially reduced species of oxygen and subsequent changes in cell metabolism. During the inflammatory phase endothelial cell injury is amplified through the release of mediators and the attraction of inflammatory cells into the lung microvasculature and into the lung interstitium.

with an accumulation of platelets in the capillary lumen. This can further augment the injury and release additional chemotactic signals to increase the influx of neutrophils. Neutrophils may act as amplifiers of inflammation in the late stages of the injury and explain why the disease process will be relatively quiescent for a large number of hours then rapidly progress to widespread destruction of the lung and death of the animal.

Modulation of Hyperoxic Injury Using Antioxidant Enzymes

Initial attempts to treat oxygen toxicity using both intraperitoneally injected superoxide dismutase and aerosolized superoxide dismutase to coat the alveolar surface gave no protection (10). The enzymes involved are large molecules which are not likely to be able to cross cell membranes. Since the major sites of superoxide production are intracelluar, injection of antioxidant enzymes into the alveolar or intravascular spaces is unlikely to provide substantial protection. Partial protection in some experimental models may be achieved if the injury is caused by activation of intravascular white cells which can produce oxygen radicals in the intravascular spaces. In *in vivo* models of pulmonary oxygen toxicity this does not appear to be the primary mechanism involved.

By encapsulating antioxidant enzymes in lipid membranes, termed liposomes, the enzymes can gain entry into the cell by at least two mechanisms. Fusion of the liposomal membrane with the cell membrane can directly release the liposome contents into the cell cytoplasm. Alternatively, endocytosis at the

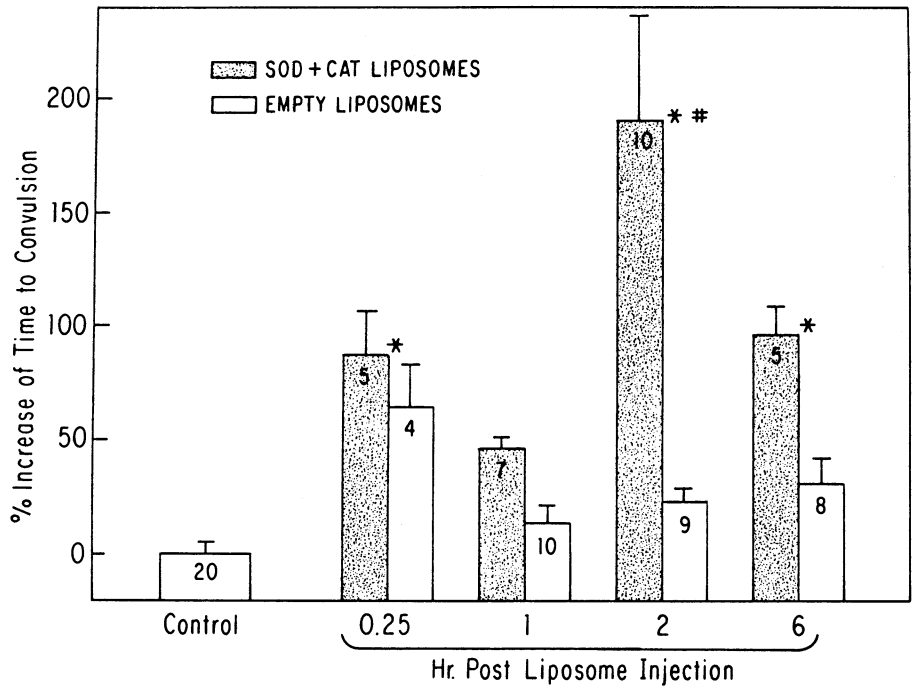

Fig. 15. Influence of superoxide dismutase (SOD) plus catalase (CAT) in empty liposomes on the time required to cause convulsions in rats exposed to 6 ATA 100% O_2. Values are expressed as percent increase of time to convulsion compared with control (24.66±1.44 min) and as means ±S.E. with n shown in the bars. Optimum results in terms of prolongation of time to convulsion occurred when the exposure to hyperbaric oxygen occurred 2 hours after the initial injection of liposomes (13). (Reproduced with permission of J Appl Physiol : Respirat Environ Physiol)

cell membrane can lead to uptake of the entire liposome and subsequent release of its contents into the cell cytoplasm.

In initial studies with liposome encapsulated antioxidant enzymes it has been shown that if the liposomes are placed over cultured endothelial cells the intracellular content of antioxidant enzymes can be increased more than sixfold (11). When these cultured cells are subsequently exposed to hyperoxia the rate of cell death, as measured by Cr^{51} release, is substantially reduced. Liposome encapsulated antioxidant enzymes have also been injected into the tail veins of rats and the animals subsequently exposed to 100% oxygen. Untreated animals die consistently after 60–72 hours exposures. Animals treated with liposomes containing both superoxide dismutase and succinolated catalase were found to survive a full 9 day exposure to 100% O_2 (12).

While injection of liposome encapsulated antioxidant enzymes into the venous system leads to delivery of these enzymes to lung capillary endothelial cells, it also can augment antioxidant enzymes in other body tissues. Yusa, et al (13) demonstrated that administration of liposome encapsulated antioxidant enzymes to rats can substantially prolong the time before the onset of seizures during exposure to hyperbaric levels of hyperoxia (**Fig. 15**). Administration of both catalase

and superoxide dismutase containing liposomes provided the highest level of protection, and more than doubled the time to seizures at two hours after the liposome treatment.

Conclusions

Hyperoxia increases oxygen radical production at a number of subcellular sites including: Mitochondria, Endoplasmic reticulum, and Nuclear membranes. This is likely to be the essential step in initiation of pulmonary oxygen toxicity.

The primary phases in the progression of pulmonary oxygen toxicity can be defined as:

(1) Initiation
(2) Inflammatory
(3) Destructive
(4) Proliferative
(5) Fibrotic

If the level of oxygen exposure is high enough, the inflammatory and destructive phases are fulminant and lead to loss of the majority of the pulmonary capillary bed and the death of the animal.

Antioxidant enyzmes, if delivered in forms that can reach the interior of the cell, can reduce the toxic effects of high concentrations of oxygen.

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