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Critical Review

Oxidative Stress, Antioxidant Defenses, and Damage Removal, Repair, and Replacement Systems

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Summary

Oxidative stress is an unavoidable consequence of life in an oxygen-rich atmosphere. Oxygen radicals and other activated oxygen species are generated as by-products of aerobic metabolism and exposure to various natural and synthetic toxicants. The "Oxygen Paradox" is that oxygen is dangerous to the very life-forms for which it has become an essential component of energy production. The first defense against oxygen toxicity is the sharp gradient of oxygen tension, seen in all mammals, from the environmental level of 20% to a tissue concentration of only 3–4% oxygen. These relatively low tissue levels of oxygen prevent most oxidative damage from ever occurring. Cells, tissues, organs, and organisms utilize multiple layers of antioxidant defenses and damage removal, and replacement or repair systems in order to cope with the remaining stress and damage that oxygen engenders. The enzymes comprising many of these protective systems are inducible under conditions of oxidative stress adaptation, in which the expression of over 40 mammalian genes is upregulated. Mitotic cells have the additional defensive ability of entering a transient growth-arrested state (in the first stages of adaptation) in which DNA is protected by histone proteins, energy is conserved by diminished expression of nonessential genes, and the expression of shock and stress proteins is greatly increased. Failure to fully cope with an oxidative stress can switch mitotic cells into a permanent growth-arrested, senescence-like state in which they may survive for long periods. Faced with even more severe oxidative stress, or the declining protective enzymes and adaptive capacity associated with aging, cells may "sacrifice themselves" by apoptosis, which protects surrounding healthy tissue from further damage. Only under the most severe oxidative stress conditions will cells undergo a necrotic death, which exposes surrounding tissues to the further vicissitudes of an inflammatory immune response. This remarkable array of systems for defense; damage removal, replacement, and repair; adaptation; growth modulation; and apoptosis make it possible for us to enjoy life in an oxygen-rich environment.

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OXIDATIVE STRESS AND THE OXYGEN PARADOX

Several million years ago, unicellular organisms in the primordial seas began dumping a new toxic agent into the environment. In a remarkable show of evolutionary adaptability, other organisms found ways to turn this toxicant to their own advantage. Many organisms developed the ability to use the toxic agent to increase energy extraction from foodstuffs, while still clinging tenaciously to archaic, but inherently safer, metabolic pathways. In time, the benefits of this toxicant permitted the emergence of new species whose very existence depended entirely on the poor waste-management practices of their primitive relatives.

The toxic agent in question, of course, is oxygen that was (and still is) produced as a metabolic waste product by simple aquatic organisms such as the blue-green algae. Not to be outdone, terrestrial plant life has mimicked its water-dwelling relatives and now contributes significantly to environmental oxygen levels. Multicellular animal life evolved partly as a consequence of the many-fold increase in cellular energy that can be extracted from foods by enzymatic reduction of oxygen. If all this seems like a perfect arrangement, it must always be remembered that appearances can be deceptive. I began this discussion by introducing oxygen as a toxic waste product and although higher animal species (obligate aerobes) cannot live without it, oxygen remains an extremely dangerous agent with which to coexist. This very basic dilemma has come to be known as "The Oxygen Paradox" (*1*, *2*).

Living with oxygen is basically unsafe. It has been said that, "A disturbance in the pro-oxidant/antioxidant systems in favour of the former may be denoted as an oxidative stress" (*3*). Thus,

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oxidative stress can result from increased exposure to oxidants or from decreased protection against oxidants, or even from both problems occurring simultaneously (*4*). Oxidation reactions cause iron to rust, potato chips to spoil, milk to go sour, and oil or meat to turn rancid. We would spoil or go rancid, too, except that we have numerous defenses to minimize oxidation. Our antioxidant defenses have been studied for many years and are known to include renewable enzymes and compounds that allow us to exist in a hostile oxygen environment. In the late 1970s and early 1980s, however, advancing research began to indicate that classical antioxidant defenses alone could not explain our high tolerance for oxygen. Simply stated, the antioxidant enzymes and compounds are not 100% effective in preventing oxidation of vital cellular constituents, and damage occurs continuously.

Estimates of damage to cells, based on laboratory experiments, indicated that we should quickly die from oxidative damage to vital cellular constituents if the antioxidant enzymes and compounds were really our only means of protection. Because death by oxidation is clearly not the norm, the existence of damage removal and repair systems was proposed. Over the past 25 years, mounting evidence from several laboratories has confirmed the existence of damage removal and repair systems for oxidized proteins, membrane lipids, and DNA. Furthermore, the effective operation of several damage removal or repair systems has been shown to be essential for survival in an oxygen-rich environment, a concept that has long been accepted for key antioxidant enzymes and compounds.

Dividing cells appear to be particularly sensitive to oxidative damage, probably because their DNA is uncoiled and naked as it undergoes rapid replication. The simple expedient of entering a transient growth-arrested state, until the oxidative stress is (hopefully) over, now appears to be a very effective protection in mitotic cells.

Perhaps one of the most exciting recent developments in the field of Oxidative Stress has been the discovery that genes, which encode antioxidant enzymes and repair enzymes, can actually respond to changing levels of oxidative insult. We now know that organisms from simple bacteria to complex mammals can successfully adapt to oxidative stress by rapidly increasing their production of antioxidant and repair enzymes. A battery of some 30–40 genes in bacteria, yeast, and mammals, are "turned on" in a rapid and highly coordinated response to oxidation. The augmented defense and repair armory provided by this rapid genetic response enables cells to survive oxidant exposures that would normally be lethal. The importance of this adaptive response in an ever-changing environment is suggested as a highly effective countermeasure against the "Oxygen Paradox" (*1*, *2*).

Oxidative stress causes a very wide spectrum of genetic, metabolic, and cellular responses. Only necrosis, which is the most extreme outcome, involves direct cell destruction. Most oxidative stress conditions that cells might actually encounter will modulate gene expression, may stimulate cell growth, or may cause a protective temporary growth-arrest and transient adaptive response. Even the apoptotic response seen at high oxidant exposures appears to protect surrounding cells and issues.

ANTIOXIDANT COMPOUNDS AND ENZYMES

Thankfully, we are not defenseless against the oxygen radicals and other activated-oxygen species to which we are constantly exposed. All aerobic organisms, including human beings, utilize a series of primary antioxidant defenses in an attempt to protect against oxidant damage.

Antioxidant Compounds

Our cells utilize a series of antioxidant compounds to directly react with oxidizing agents and disarm them (*5–7*). Such antioxidants are said to be "scavengers," and their role is unavoidably suicidal. Vitamin E (α-tocopherol) is a major membrane-bound antioxidant, and vitamin C (ascorbic acid) is a major aqueous-phase antioxidant. The fact that these compounds are both human vitamins underscores their vital importance in maintaining health.

Other water-soluble antioxidant compounds include uric acid, glutathione, and ceruloplasmin. Uric acid, the end product of purine metabolism in humans, is particularly interesting because it may function both as a classic suicidal antioxidant and as a chelator of transition metals. By binding iron and/or copper, uric acid may inhibit metal-catalyzed oxidation reactions without itself becoming oxidized. Other lipid-soluble agents, including β-carotene and ubiquinone, may also play important antioxidant roles in vivo.

Antioxidant Enzymes and Proteins

Aerobic organisms also synthesize numerous antioxidant enzymes and other proteins in an attempt to minimize oxidative damage (*6*, *7*). Perhaps the best known of these enzymes is superoxide dismutase. No single discovery was of greater significance to the development of the free-radical field than the discovery of the enzyme family of superoxide dismutases. The superoxide dismutases (SOD) catalyze the reaction $O_2^{\cdot-} + O_2^{\cdot-} \rightarrow H_2O_2 + O_2$ and increase the rate constant of this important dismutation reaction several-fold. All members of the SOD family utilize transition metals at their active sites. Bacteria employ an Fe-SOD and a Mn-SOD, whereas mammals utilize distinct cytoplasmic and extracellular forms of Cu, Zn-SOD and a mitochondrial Mn-SOD that, in evolutionary terms, is closely related to the bacterial Mn-SOD. Genetic deletion of SOD has been shown to be a lethal mutation in lower organisms, underpinning the essential importance of this enzyme family.

The product of SOD is H_2O_2, which is clearly toxic and must be rapidly removed. In mammalian cells this is accomplished by two enzyme families: the glutathione peroxidases and the catalases. Both glutathione peroxidases and catalases detoxify H_2O_2 by reducing it to water and oxygen. Glutathione peroxidases utilize the reducing power of glutathione (GSH), a tripeptide consisting of L-γ-glutamyl-L-cysteinylglycine, to detoxify hydrogen peroxide. The sulfhydryl moiety of the cysteine

residue supplies the actual reducing equivalents required for glutathione peroxidase activity. Two molecules of GSH are oxidized to form the disulfide-bonded compound, GS–SG in the reduction of a molecule of hydrogen peroxide. The companion enzyme glutathione reductase utilizes NADPH to re-reduce one molecule of GS–SG to two molecules of GSH, thus permitting the continuous action of glutathione peroxidase. So important are the roles of glutathione-utilizing enzymes to normal functions that most cells contain concentrations of GSH in excess of 5 mM.

A good deal of interest has centered on the question of whether glutathione peroxidases or catalases are more important in detoxifying intracellular hydrogen peroxide. In most mammalian cell types, catalase is exclusively found within peroxisomes where it has a clear function of removing the H_2O_2 generated by β-oxidation of long-chain fatty acids. Because catalase is not generally found in the cytoplasm of most mammalian cells, and because bulk H_2O_2 diffusion from the cytoplasm into peroxisomes seems rather unlikely, it seems probable that glutathione peroxidases largely deal with cytoplasmic H_2O_2 and catalases largely deal with peroxisomal H_2O_2.

Another important antioxidant enzyme is DT diaphorase, which is also called quinone reductase. DT diaphorase is able to catalyze the direct divalent reduction of many (dehydro)quinones to (dihydro)quinols. By catalyzing a direct two-electron reduction of substrate quinones, DT diaphorase avoids production of reactive semiquinone radical intermediates (such as $Q^{\bullet -}$ and $QH^{\bullet}$). DT diaphorase may play an important role in the detoxification of many quinonoid drugs and environmental agents by stabilizing the relatively safe quinol form, prior to conjugation and elimination by other enzyme systems.

Two nonenzymatic proteins, ferritin and ceruloplasmin, also appear to play important roles in transition-metal storage and antioxidant defense in vivo. Transition metals such as iron and copper are involved in both metal-catalyzed (“auto”)oxidations and reactions leading to hydroxyl radical production from superoxide. Ferritin, which binds iron in the cytoplasm of mammalian cells, and ceruloplasmin, which binds copper in plasma, are thought by many to contribute a significant antioxidant capacity to bodily fluids. Recently, despite the enormity of the task, significant progress has been made in detailed and quantitative analysis of the total antioxidant status of various bodily fluids.

TRANSIENT GROWTH-ARREST

One of the surprises to emerge from studies of cellular stress responses is the observation that transient growth-arrest is utilized by mitotic cells to protect against acute stress (*1*, *8–14*). Our current understanding or interpretation is that the DNA of dividing cells is much more susceptible to damage than is that of postmitotic cells. In postmitotic cells, DNA is, for the most part, maintained in a supercoiled state and is further protected by a coating of histone proteins. In contrast, the DNA of mitotic cells spends long periods of time in an uncoiled state, with no histone protein coating, in order to undergo replication. This uncoiled and naked state of DNA in dividing cells makes them especially susceptible to various forms of damage, including oxidative stress. The situation is further exacerbated by the heavy metabolic demands of very high rates of transcription, translation, and the multiple processes leading to division in mitotic cells. Thus, an early response of mitotic cells to oxidative stress is to enter a transient growth-arrested state in which DNA is largely supercoiled, replication is halted, and only a few shock or stress genes are transcribed and translated (*8–14*). If the oxidative stress is not severe enough to cause apoptosis or necrosis, mammalian cells will re-enter the growth cycle after some 3 to 4 hr of transient growth-arrest.

It is interesting to note that transient growth-arrest has been overlooked as a protective mechanism for many years. This is probably due to the widespread popularity of cell proliferation assays as measures of toxicity. There are probably thousands of published studies in the literature in which diminished cell proliferation is used as a measure of toxicity. In many cases of high oxidant stress, diminished proliferative capacity probably does accurately reflect toxicity. In cases of mild stress, however, cells entering a protective transient growth-arrest will often be inadvertently included with permanently growth-arrested cells, or even dead cells. As will be seen in the next section on adaptive responses and inducible defenses, transiently growth-arrested cells re-enter the growth cycle, undergo adaptive changes, and actually exhibit increased tolerance to oxidative stress after several hours.

At least three genes have been shown to be important in oxidant-induced transient growth-arrest. These are *gadd45*, *gadd153* discovered by Fornace and Holbrook (*12–14*), and *adapt 15*, discovered in our laboratory (*10*, *11*). Expression of these genes, by several methods, has been shown to induce transient growth-arrest and to temporarily increase protection against oxidative stress.

DIRECT REPAIR SYSTEMS

Damage repair systems may be classified as either direct or indirect (*1*, *2*, *15*). Direct repair, about which we know only a little, has so far only been demonstrated for a few classes of oxidized molecules. One important direct repair process is the re-reduction of oxidized sulfhydryl groups on proteins. Cysteine residues in proteins are highly susceptible to autooxidation and/or metal-catalyzed oxidation. When two nearby cysteine residues within a protein oxidize, they often form a disulfide bond, producing a more rigid protein. Disulfide bonds can also form between two proteins, promoting the formation of large supramolecular assemblies of inactivated enzymes and proteins; this is called intermolecular cross-linking. Both intramolecular disulfide cross-links and intermolecular disulfide cross-links can be reversed to some extent by disulfide reductases within cells. Our understanding of such enzymatic reactions is still at an early stage. Another important sulfhydryl oxidation process is the oxidation of methionine residues to methionine sulfoxide, which may cause loss of enzyme/protein function, or which may protect

other amino acid residues in the protein. The enzyme methionine sulfoxide reductase can regenerate methionine residues within such oxidized proteins and restore function. As with disulfide reductases, our understanding of methionine sulfoxide reductases is still in its infancy.

Direct repair of DNA hydroperoxides by glutathione peroxidase has been reported from in vitro studies. The extent to which DNA peroxides are actually formed in vivo, however, is not completely clear. Also, not yet studied is the extent to which DNA peroxides may be directly repaired by glutathione peroxidases in vivo. Other relatively straightforward mechanisms of DNA repair are also being explored.

DAMAGE REMOVAL AND REPLACEMENT/REPAIR SYSTEMS (INDIRECT REPAIR)

Although our knowledge of direct repair systems, as outlined previously, is still rather rudimentary, a great deal more is known about indirect repair systems. Indirect repair involves two distinct steps; first the damaged molecule (or the damaged part of a molecule) must be recognized and excised, removed, or degraded. Next, a replacement of the entire damaged molecule must be synthesized, or the excised portion of the damaged molecule must be made and inserted.

Degradation and Replacement of Oxidized Proteins

Extensive studies have revealed that oxidized proteins are recognized by proteases and completely degraded (to amino acids); entirely new replacement protein molecules are then synthesized de novo (*15–20*). It appears that oxidized amino acids within oxidatively modified proteins are eliminated or used as carbon sources for ATP synthesis. Because an oxidatively modified protein may contain only two or three oxidized amino acids, it appears probable that most of the amino acids from an oxidized and degraded protein are reutilized for protein synthesis. Thus, during oxidative stress many proteins synthesized as damage replacements are likely to contain a high percentage of recycled amino acids. During periods of particularly high oxidative stress, the proteolytic capacity of cells may not be sufficient to cope with the number of oxidized protein molecules being generated. A similar problem may occur in aging, or with certain disease states, when proteolytic capacity may decline below a critical threshold of activity required to cope with normal oxidative stress levels. Under such circumstances, oxidized proteins may not undergo appropriate proteolytic digestion and may instead cross-link with one another or form extensive hydrophobic bonds. Such aggregates of damaged proteins are detrimental to normal cell functions and lead to further problems.

In bacteria such as *Escherichia coli*, a series of proteolytic enzymes act cooperatively in the recognition and degradation of oxidatively modified soluble proteins. A similar series of proteolytic enzymes appear to conduct the degradation of oxidatively modified soluble proteins in mammalian mitochondria. In the cytoplasm and nucleus of eukaryotic cells, however, oxidized soluble proteins largely appear to be recognized and degraded by the proteasome complex, which constitutes up to 1% of total cellular protein. Proteasome is a 670-kDa multi-enzyme complex that appears to be ubiquitously expressed in the cytoplasm and nuclei of all eucaryotic cells. Some 14 individual polypeptides, each present in multiple copies, with molecular weights ranging from approximately 20,000 daltons to 35,000 daltons make up the proteasome complex; the exact composition varies with species and cell type. Each of the component proteasome polypeptides is encoded by a separate gene and many of these genes have now been cloned and sequenced. The results of such cloning and sequencing studies reveal that proteasome is a completely nonclassical protease complex. Indeed, the proteasome subunits have no discernable sequence identity to any known proteins, except for a small degree of sequence overlap with some of the heat shock proteins.

Recognition of oxidized soluble proteins in the cell cytoplasm and nucleus by proteasome appears to occur via binding to exposed hydrophobic patches on the damaged proteins. Although the process of protein oxidation (which, of course, means oxidation of consistent amino acids) often involves changes that make some amino acid residues more hydrophilic; changes in charge relationships on a protein can cause significant unfolding or partial denaturation. Such partial denaturation exposes previously shielded stretches of primary sequence that are hydrophobic in nature. Exposed hydrophobic patches on the surface of oxidized proteins appear to act as recognition and binding sequences for the 670-kDa core proteasome. Several lines of research now indicate that gradually increasing proteasome inhibition, from accumulation of nondegradable protein aggregates, may be a significant factor in the overall aging process (*20*).

Degradation and Replacement/Repair of Oxidized Membrane Lipids

Lipid peroxidation was the first type of oxidative damage to be studied. Membrane phospholipids are continually subjected to oxidant challenges. The process of lipid peroxidation is comprised of a set of chain reactions that are initiated by the abstraction of a hydrogen atom (from carbon) in an unsaturated fatty acyl chain (*1–4*). In an aerobic environment, oxygen will add to the fatty acid at the carbon centered lipid radical (L•) to give rise to a lipid peroxyl radical (LOO•). Once initiated, LOO• can further propagate the peroxidation chain reaction by abstracting a hydrogen atom from other vicinal unsaturated fatty acids. The resulting lipid hydroperoxide (LOOH) can easily decompose into several reactive species including: lipid alkoxyl radicals (LO•), aldehydes (e.g., malonyldialdehyde), alkanes, lipid epoxides, and alcohols. Cholesterol has also been shown to undergo oxidation, to give rise to a variety of epoxides and alcohols.

Peroxidized membranes become rigid, lose selective permeability, and under extreme conditions, can lose their integrity. Water-soluble lipid peroxidation products (most notably the aldehydes) have been shown to diffuse from membranes into other subcellular compartments. Dialdehydes can act as cross-linking reagents and are thought to play a role in the protein

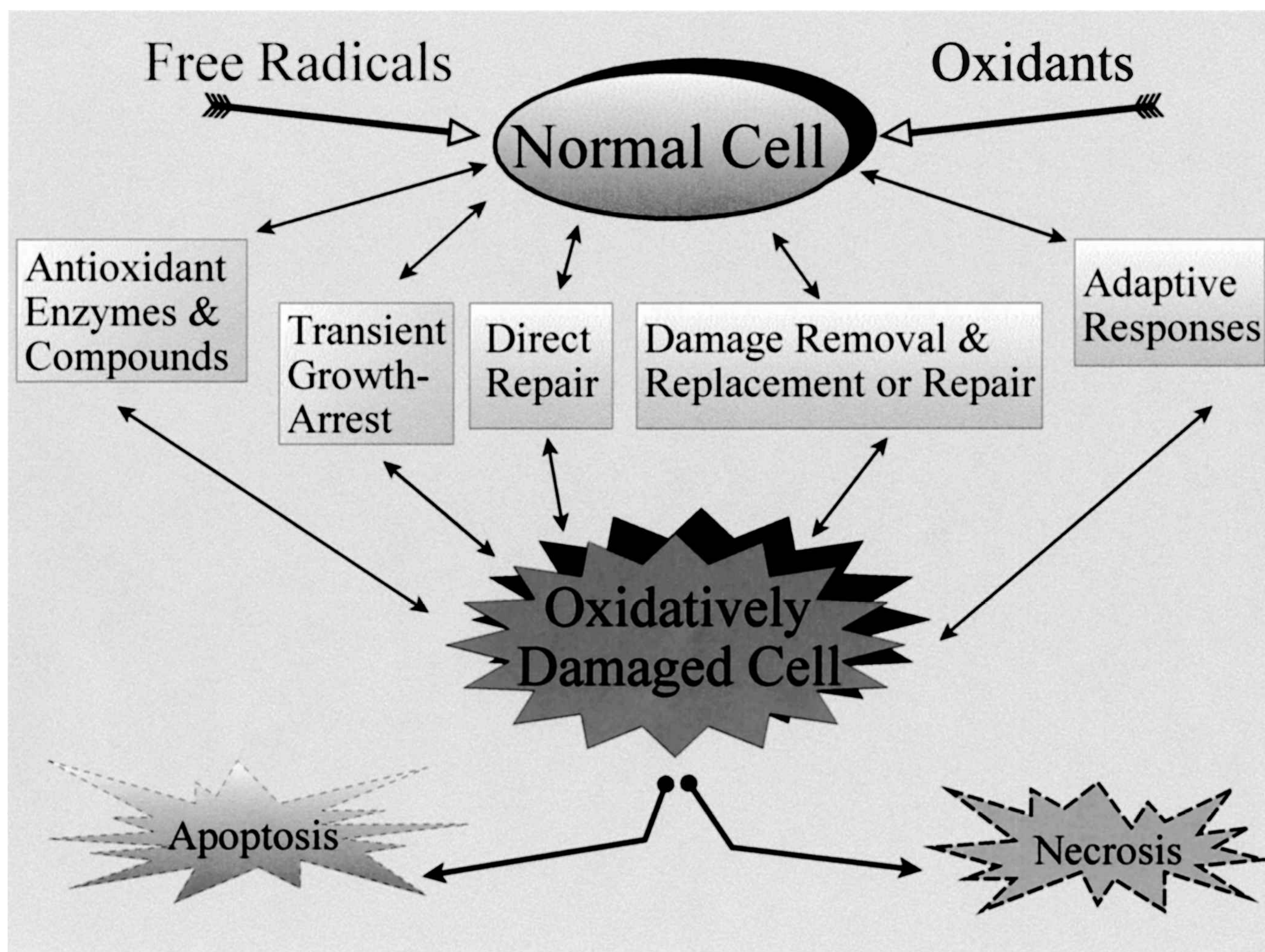

Figure 1. Oxidative stress, antioxidant defenses, and damage removal, repair, and replacement systems. Depicted here is the conversion (by free radicals or other oxidants) of a normal, mitotic, eukaryotic cell into an oxidatively damaged cell, which may then die by either apoptosis or necrosis. Acting against the conversion of a normal cell into an oxidatively damaged cell are the antioxidant enzymes and compounds (primary defenses) and the facility of mitotic cells to enter a protective transient growth-arrested state. Should these protections not prove sufficient, some of the damaged proteins, lipids, and DNA may undergo direct repair, and other damaged proteins, lipids, and DNA will be partially or completely degraded and then repaired or completely replaced. While repair, removal, and replacement mechanisms are underway, cells will begin a series of temporary adaptive responses involving altered expression of at least 30–40 genes. Maximal adaptation (greatest induced resistance to oxidative stress) is typically seen at 18–20 hr after stress exposure, and lasts for no more than 36 hr. If these multiple layers of defense, repair, removal, replacement, and adaptation are insufficient to deal with an oxidative stress, the normal cell will become an oxidatively damaged cell. The damaged cell may enter a permanently growth-arrested state from which it never truly recovers, or it may die by apoptosis, which will protect surrounding cells and tissues. The other option of necrotic cell death, which typically only occurs at very high oxidative stress levels, involves loss of membrane integrity and release of cellular constituents, which will cause an inflammatory immune response that may also damage adjacent cells.

aggregation that forms the age pigment, lipofuscin. Several laboratories are investigating the possibility that lipid peroxidation products may form DNA adducts, thus giving rise to mutations and altered patterns of gene expression. Others have noted inhibition of enzyme function by lipid peroxidation products. It is very clear that the process of lipid peroxidation, and its products, can be detrimental to cell viability. Cumulative effects of lipid peroxidation have been implicated as underlying numerous pathological conditions including: atherosclerosis, hemolytic anemias, and ischemia reperfusion injuries.

Lipid bilayers that have been oxidized become better substrates for phospholipase enzymes (*15*, *21*). Phospholipase A_2 acts at the sn-2 position of the phospholipid glycerol backbone to generate a free fatty acid and a lysophospholipid. Phospholipase

A_2 has been shown to preferentially hydrolyze fatty acids from oxidized liposomes (*21*). Structural perturbations due to changes in membrane microviscosity, and the increased hydrophilic nature of oxidized lipids may be responsible for the increased susceptibility to phospholipase A_2 action. Removing fatty acid hydroperoxides from the membrane compartment will help prevent further propagation reactions. Additionally, it has been demonstrated that fatty acid hydroperoxides released into the cytosol are substrates for glutathione peroxidase. Glutathione peroxidase detoxifies fatty acid hydroperoxides by reducing them to their corresponding hydroxy fatty acids. Lysophospholipids left in the membrane posses potential detergent properties that have been shown to disrupt membrane structure and function. Lysophospholipids can serve as substrates for reacylation reactions (readdition of fatty acids to the sn-2 position) to regenerate intact phospholipids.

Recent work suggests that it is possible to reduce fatty acid hydroperoxides (to their corresponding alcohols) without hydrolysis and release from the membrane compartment. A member of the glutathione peroxidase family, phospholipid hydroperoxide glutathione peroxidase, which acts preferentially on phospholipid hydroperoxides has been characterized by Ursini, Maiorino, and their coworkers (*22*). A glutathione transferase with activity towards lipid hydroperoxides has also been extracted from nuclei.

Peroxidized membranes and lipid oxidation products represent a constant threat to aerobic cells. It is now widely held that in addition to preventing initiation of peroxidation (with compounds such as vitamin E), cells have also developed a variety of mechanisms for maintaining membrane integrity and homeostasis by repairing oxidatively damaged lipid components.

Repair of Oxidized DNA

Ribo- and deoxyribonucleic acids are also vulnerable to oxidative damage, and perhaps most importantly, DNA has been shown to incur oxidative damage in vivo (*1–4*). Although DNA is a relatively simple biopolymer made up of only four different nucleic acids, its integrity is vital to cell division and survival. Oxidative alterations to nucleic acid polymers has been shown to disrupt transcription, translation, and DNA replication, and to give rise to mutations and (ultimately) cell senescence or death. Despite the precious nature of the genetic code, it also appears to be a target for oxidative damage. The amount of oxidative damage, even under normal physiological conditions, may be quite extensive with estimates as high as 1 base modification per 130,000 bases in nuclear DNA. Damage to mitochondrial DNA is estimated to be even higher at 1 per 8,000 bases. Fragments of oxidatively modified mitochondrial DNA have been implicated in cancer and aging.

Oxidants can elicit a wide variety of DNA damage products, several of which have been carefully characterized. The types of DNA damage can be grouped into: strand breaks (single and double), sister chromatid exchange, DNA-DNA and DNA-protein cross-links, and base modifications. All four DNA bases can be oxidatively modified; however, the pyrimidines (cytosine and, especially, thymidine) appear to be most susceptible. Bases undergo ring saturation, ring opening, ring contraction, and hydroxylation. These types of alterations usually result in a loss of aromaticity and planarity, which can cause local distortions in the double helix. Depending on the type and extent of damage, the altered bases can be found either attached to, or dissociated from the DNA molecule to generate apurinic/apyrimidinic (AP) sites.

Radical interactions with DNA appear to be fairly nonspecific; hence, the phosphodiester backbone may also be oxidatively damaged. Damage to the sugar and phosphate moieties, which form the backbone, may result in strand breaks. Depending on the site of radical attack, unusual 3′ and 5′ ends (i.e., non-3′-OH, non-5′-PO_4) can be generated. These abnormal ends are not substrates for DNA polymerases, and must be removed before any repair can occur.

Reports of glutathione transferases and peroxidases using thymidine hydroperoxide as a substrate have been published (*23–26*). DNA may also undergo oxidative demethylation (*23–26*). DNA methylases may play an important role in restoring methylation patterns and maintaining epigenetic phenomena. Inhibition of poly (ADP-ribose) polymerase has been shown to exacerbate H_2O_2 genotoxicity, although the mechanism for this is not yet clear. There is ample evidence that several prokaryotic and eukaryotic enzymes repair oxidatively damaged DNA by both direct (*23–26*) and excision repair mechanisms (*23–26*).

Prokaryotic endonuclease III has been shown to cleave at the 3′ side of AP sites. Endonuclease III also appears to posses an *N*-glycosylase activity for thymine glycol and urea residues; two common products of oxidative damage to DNA. Exonuclease III possess a 3′- to 5′-nucleolytic activity, which may be responsible for removing the sugar fragments generated during oxidative strand breakage. Exonuclease III is really a poor name for this enzyme, which actually expresses 85% of the 5′ AP endonucleolytic activity in *E. coli*. Endonuclease IV is also a 5′-AP endonuclease.

Several eukaryotic glycosylases that act on DNA oxidation products have been characterized. A 3′-repair diesterase in yeast is apparently responsible for removing damaged 3′ termini left by free radical reactions. A mammalian endonuclease has been isolated based on its specificity for oxidatively modified DNA. It bears remarkable similarities to the bacterial endonuclease III including: molecular weight (~30 kDa), lack of divalent cation requirement, and substrate specificity. The term “redoxyendonucleases” has been proposed for all nucleases that participate in repairing oxidatively damaged DNA.

There is substantial evidence for the vital role of redoxyendonucleases in higher eucaryotic cells. DNA damage that appears in cells as a result of an acute oxidant challenge (including base damage and single-strand breaks) has been shown to diminish as a function of time. These results suggest that a removal of lesions is being carried out by intracellular systems. Oxidatively damaged bases (8-hydroxydeoxyguanosine, thymine glycol, and

thymidine glycol) have been measured in animal urine. Again, this suggests that there is systematic excision and excretion of oxidized DNA in vivo. The vital importance of such DNA excision repair processes was highlighted by the selection of DNA repair as the "Molecule of the Year" for 1994 by *Science* magazine.

ADAPTIVE RESPONSES AND INDUCIBLE DEFENSES

One of the most exciting developments in the field of oxidative stress over the past two decades has been the discovery that both prokaryotes and eukaryotes are able to dramatically upregulate their armory of oxidant protections in response to an oxidative stress. Many researchers have utilized a fairly common cell-culture adaptive response protocol originally used in heat-shock studies to study such phenomena. This approach involves first finding an oxidant concentration that is lethal to most of the cells. Next new cultures are exposed to much lower levels of the same oxidant (pretreatment exposures) for various periods of time before being exposed to the normally lethal concentration (the challenge dose). It has now been widely found that, in cells from *E. coli* to human hepatocytes, pretreatment conditions can be found that will enable cells to survive the subsequent challenge dose. Such adaptive responses to oxidative stress have been shown to involve widespread alterations in gene expression.

Vital early studies on temporary adaptation to oxidative stress were performed by Spitz et al. (*27*) and by Laval (*28*). After 3–5 hr of transient growth-arrest, many cells exposed to mild oxidative stress (e.g., μM levels of hydrogen peroxide) undergo further changes that can be characterized as temporary adaptation to oxidative stress. In mammalian fibroblasts, we (*9–11*, *29–34*), and others (*27*, *28*) have studied, maximal adaptation is seen approximately 18 hr after initial exposure to hydrogen peroxide; i.e., some 13–15 hr after they exit from transient growth-arrest. In bacteria such as *E. Coli* and *Salmonella*, maximal adaptation is seen 20–30 min after oxidant exposure (*29*, *35*, *36*), whereas yeast cells require some 45 min for maximal adaptation (*29*, *37*).

It is important to note that the adaptation referred to in this section simply means increased resistance to oxidative stress, as measured by cell proliferation capacity. Furthermore, the adaptation is temporary, lasting some 18 hr in mammalian fibroblasts, 90 min in yeast, and only 60 min in *E. coli*. In our studies, we have been especially careful to avoid selecting for pre-existing resistant cell in the population by checking repeatedly that transiently adapted cells can actually de-adapt.

In both prokaryotes and eukaryotes, temporary adaptation to oxidative stress depends on transcription and translation. A large number of genes undergo altered expression during the adaptive response. Some genes are upregulated, some are downregulated, some are modulated early in the adaptation, while the expression of others is affected at later times. In mammalian fibroblasts, we observe three broad "waves" of altered gene expression during adaptation; one at 0–4 hr following H_2O_2 exposure (which is the expression of shock/stress genes during transient growth-arrest), one at 4–8 hr, and one at 8–12 hr. Inhibiting either transcription or translation during the adaptive response greatly limits the development of increased H_2O_2 resistance. If both transcription and translation are inhibited, little or no adaptation will occur. Therefore, the transient adaptive response to oxidative stress depends largely on altered gene expression but partly on increased translation of pre-existing mRNAs. It further appears that message stabilization (for some mRNAs), increased message degradation (for other mRNAs), and altered precursor processing are all involved in adaptive translational responses (*1*, *9–11*, *29–34*, *37*).

Elegant studies in *E. coli* and *Salmonella* have shown that two particular regulons are responsible for many of the bacterial adaptive responses to oxidative stress: the oxyR regulon (*38*) and the soxRS regulon (*39*). In mammalian cells, no "master regulation molecules" have been found, but at least 40 gene products are involved in the adaptive response. Several of the mammalian adaptive genes are involved in antioxidant defenses and others are damage removal or repair enzymes. Many classic shock or stress genes are involved very early in adaptive responses, particularly during transient growth-arrest. As indicated earlier in this review, transient growth-arrest is a very important early portion of the early defensive response of divisionally competent mammalian cells to oxidative stress. We now know that transient growth-arrest itself is a very early part of adaptive responses, and that the genes *gadd153*, *gadd45*, and *adapt15* play important roles in inducing transient growth-arrest (*9*, *10–14*).

The transcription factor, adapt66 (a *mafG* homologue) is probably responsible for inducing the expression of several other adaptive genes and for coordinating expression of several mitochondrial proteins, the subunits of which are encoded by both the nuclear genome and the mitochondrial genome (*31*). A number of other "adapt" genes have recently been discovered but their functions are not yet clear. One of these genes is the calcium-dependent *adapt33* (*30*), and another is *adapt73*, which appears to also be homologous to a cardiogenic shock gene called *PigHep3* (*32*). *Adapt 78* (which is also called *DSCR1*) is strongly induced during oxidative stress adaptation (*33*, *34*). *Adapt 78* contains a calcium response element, is highly inducible by calcium ionophores alone, and may well be involved in protecting against the harmful effects of large calcium fluxes during oxidative stress. More recent work indicates that *adapt78* may also involved in Down Syndrome, Parkinson's Disease, and Alzheimer's Disease (*40*).

Numerous other genes have been shown to be inducible in mammalian cell lines following exposure to the relatively mild level of hydrogen peroxide oxidative stress that we find will cause transient adaptation. These include the protooncogenes c-*fos* and c-*myc* (*41*), c-*jun*, *egr*, and JE (*41–43*). Similar oncogene induction has also been reported following exposure to *tert*-butylhydroperoxide (*43*). The induction of heme oxygenase by many oxidants, including mild peroxide stress, may have a strong protective effect, as proposed by Keyse and Tyrrell (*44*). Other gene products that have been reported to be induced by relatively mild hydrogen peroxide stress in dividing

mammalian cell cultures include: the CL100 phosphatase (*45*); interleukin-8 (*46*); catalase, glutathione peroxidase, and mitochondrial mangano-superoxide dismutase (*47*); natural killer-enhancing factor-B (*48*); mitogen-activated protein kinase (*49*); and gamma-glutamyltranspeptidase (*50*). Relatively low levels of nitric oxide have also been shown to induce expression of c-*jun* (*51*), c-*fos* (*51*, *52*), and zif/268 (*52*). The list of oxidant stress-inducible genes is much longer than the space limitations of this review article will allow; apologies are extended to those investigators whose studies have not been cited here. It is, however, very important to note that many of the gene inductions reported in this work have not actually been studied in an adaptive cell culture model. Thus, although many of the genes discussed here appear to be excellent candidates for involvement in transient adaptation to oxidative stress, their actual importance remains to be tested.

It is important to note that, although most work on adaptation has been conducted in dividing cells (largely because of the simplicity of proliferative studies), postmitotic cells also exhibit significant protective adaptive responses to oxidative stress (*15*). Although such adaptive responses do not include transient growth-arrest (postmitotic cells are permanently growth-arrested), they do include increased expression of various antioxidant enzymes, DNA repair enzymes, and other protective proteins. Thus, adaptation to oxidative stress must also be an important component of the defensive/protective repertoire of cells in vital organs such as the brain, heart, and skeletal muscle.

In concluding this section, a note must be made of important studies involving permanent (or stable) oxidative stress resistance. Investigators have chronically exposed cell lines to various levels of oxidative stress over several generations, and have selected for pre-existing or mutant phenotypes that confer oxidative stress resistance. Several such studies have reported dramatic increases in catalase activity (relative to the parent population), such as the 20-fold higher levels reported by Spitz et al. (*53*). Stable oxidative stress resistance may tell us a great deal about the importance of individual genes to overall cellular survival, and the value of such cell lines should not be underestimated. It should be clear, however, that transient adaptive responses in gene expression and stable stress resistance are quite different entities.

APOPTOSIS PROTECTS SURROUNDING CELLS

A fraction of cells exposed to high levels of oxidative stress will enter the apoptotic pathway. The mechanism of oxidative stress-induced apoptosis appears to involve loss of mitochondrial transmembrane potential (*54*), release of cytochrome *c* to the cytoplasm (*55*), loss of bc1-2 (*56*), downregulation and degradation of mitochondrially encoded mRNA, rRNA, and DNA (*57–59*), and diminished transcription of the mitochondrial genome (*60*). Current thinking about toxicant-induced apoptosis suggests that, in multicellular organisms, the repair of severely damaged cells represents a major drain on available resources. To avoid this difficulty, it is suggested, individual cells within organisms (or organs or tissues) will "sacrifice" themselves for the common good of the many.

Apoptotic cells are characterized by "blebbing," nuclear condensation, and DNA laddering (*61*). Such cells are engulfed by phagocytes that prevent an immune reaction and recycle usable nutrients (*62*, *63*). Under high oxidative stress, some cells will simply disintegrate or become necrotic. Unfortunately, badly damaged cells that die by necrosis cause major immune and inflammatory responses, which may cause further damage to surrounding cells and tissues. Such secondary inflammation (also an oxidant stress) may be particularly important in many autoimmune diseases such as rheumatoid arthritis and lupus. Oxidation-induced necrosis may also play a significant role in ischemia-reperfussion injures such as heart attacks, strokes, ischemic bowel disease, and macular degeneration. Thus, for a tissue, organ, or organism, removing badly damaged cells by apoptosis represents a very real advantage (over necrosis) and should be considered as one of the "defense mechanisms."

The apoptotic pathway may be very important in several age-related diseases such as Parkinson's, Alzheimer's, and sarcopenia. Importantly, many mitochondrial changes, including loss of membrane potential (*54*) and downregulation and degradation of mitochondrial polynucleotides (*57–59*), are common to apoptosis directly induced by oxidants and to apoptosis induced by staurosporine or IL-2 withdrawal. Furthermore, over expression of the p53 gene has been seen to result in induction of multiple "redox-related" gene products, and initiation of apoptosis (*62*). These observations support a strong involvement of oxidative stress mechanisms in general apoptotic pathways.

THE IMPORTANCE OF OXYGEN CONCENTRATION

Most laboratory studies of cells in culture have been, and still are, conducted in open conditions that expose the cells to atmospheric levels of approximately 20% oxygen. The only cells in human or animal bodies to experience such high oxygen levels are the lens cells of the eyes, and cells of the upper airways and upper regions of the lungs. Most mammalian cells exist in vivo at an oxygen tension of 4% or less; many would argue that 2% is a better physiological estimate. In humans, there is, for example, a gradient of oxygen tension (partial pressure) decreasing from 150 mm Hg in the environment, to aproximately 110 mm Hg in the lungs, to 95 mm Hg in the arterial system, down to only 30 mm Hg in most tissues (*64*). The main tissue that experiences dramatic changes in oxygen delivery in vivo is the skeletal muscle, which during heavy exercise can undergo up to 100-fold increases in blood flow. Interestingly, heavy exercise has been shown to exert an oxidative stress in skeletal muscle (*65*, *66*). Thus, almost all cell culture studies that one reads are actually experiments in oxidative stress!

Cultured cells grown in 20% oxygen are essentially pre-adapted or preselected to survive under conditions of oxidative

stress. If cells are instead grown in 3% oxygen, much closer to physiological cellular levels, they are more sensitive to an oxidative challenge but exhibit far less accumulated oxidant damage. Measures of accumulated protein oxidation, lipid oxidation, and oxidative DNA damage are all lower in cells grown at 3% O_2 than in cells grown at 20% oxygen. When comparing cells grown at 3% O_2 with those grown at 20% O_2, we find that the 3% O_2 cells have quite low antioxidant enzyme levels (and are sensitive to relatively low levels of H_2O_2) but exhibit dramatic adaptive responses in the expression of defense, repair, and *adapt* genes if exposed to adaptive levels of hydrogen peroxide. In contrast, cells grown at 20% O_2 already have high levels of antioxidant enzyme activity (presumably so they can survive the 20% O_2) and exhibit much smaller adaptive increases in gene expression than do cells grown at 3% O_2 (*8–11*, *29–34*, *40*).

Interestingly, if one goes to the other extreme of growing cells at 40% O_2 (hyperoxia), one finds clear-cut examples of overt oxidative damage and stress (*20*, *67*, *68*). Thus, cells grown at 40% O_2 accumulate extensive protein aggregates, display compromised proteolysis and proteasome activity, and exhibit a senescence-like phenotype within 8–12 weeks. Of course, we have long known that the toxicity of hyperbaric "normoxia" is due to the increased oxygen solubility at high pressures, which causes tissues to undergo oxidation-induced damage.

It seems appropriate to consider oxygen concentration or tension as a continuum. Physiological concentrations of 2–4% O_2 appear to be well-tolerated by eukaryotic cells that keep in reserve a tremendous adaptive capacity for antioxidant, repair, and *adapt* gene expression under these conditions. If grown at 20% O_2, cells significantly increase their expression of antioxidant, repair, and *adapt* genes but eventually begin to exhibit signs of oxidative stress, damage accumulation, and diminished function. At 40% O_2 (or under "normoxic" hyberbaric conditions), both the defensive and adaptive capacities of cells are overwhelmed and significant accumulation of oxidatively damaged proteins, lipids, and DNA occurs, resulting in early senescence or cell death.

An important conclusion from such observations is that we have, heretofore, almost certainly underestimated the in vivo cellular capacity to cope with oxidative stress. The limited adaptive capacity of cells grown at 20% O_2 (where antioxidant, damage removal, and repair activities may already be almost maximized for survival) can now clearly be seen as reflecting only the last 20% of adaptive capabilities. Despite the increased technical difficulties of performing cell culture at only 3–4% oxygen, it is important for many of our assumptions about oxidative stress to be re-examined at physiological oxygen levels. Furthermore, the term "normoxia" should be used very carefully with respect to living organisms. Although seems entirely appropriate to describe 20% oxygen as "environmental normoxia," we must always remember that "tissue normoxia" really involves only 3–4% oxygen.

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