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The Spectrum of Responses of Oxidants in Proliferating Cells: Concentration, Concentration, Concentration

Kelvin J.A. Davies, Ph.D.

James E. Birren Chair and Assoc. Dean for Research
Ethel Percy Andrus Gerontology Center
University of Southern California
3715 McClintock Avenue, Room 306
Los Angeles, California 90089-0191
Tel: 213/740-8959
Fax: 213/740-6462
Email: Kelvin@usc.edu

Overview

Proliferating mammalian cells exhibit a broad spectrum of responses to oxidative stress, depending on the stress level encountered. Very low levels of hydrogen peroxide, for example 3 to 15 µM, or 0.1 to 0.5 µmol/10^7 cells, cause a significant mitogenic response with 25 to 45% growth stimulation. Higher H_2O_2 concentrations of 120 to 150 µM, or 2 to 5 µmol/10^7 cells, cause a temporary growth-arrest that appears to protect cells from excess energy usage and DNA damage. After 4-6 hours of temporary growth-arrest many cells will exhibit up to a 40-fold transient adaptive response in which genes for oxidant protection and damage repair are preferentially expressed. After some 18 hours of H_2O_2 adaptation (including the 4-6 hours of temporary growth arrest) cells exhibit maximal protection against oxidative stress. The H_2O_2 originally added is metabolized within 30-40 minutes and if no more is added the cells will gradually de-adapt, so that at 36 hours after the initial H_2O_2 stimulus they have returned to their original level of H_2O_2 sensitivity. At levels of H_2O_2 of 250 to 400 µM, or 9 to 14 µmol/10^7 cells, mammalian fibroblasts are not able to adapt, but instead enter a permanently growth-arrested state in which they appear to perform most normal cell functions, but never divide again. This state of permanent growth-arrest has often been confused with "cell death" in toxicity studies that have relied solely on cell proliferation assays as measures of viability. If the oxidative stress level is further increased to 0.5 to 1.0 mM H_2O_2, or 15 to 30 µmol/10^7 cells, apoptosis results. This oxidative stress-induced apoptosis involves nuclear condensation, loss of mitochondrial transmembrane potential, degradation/down regulation of mitochondrial mRNA's and rRNA's, and degradation/laddering of both nuclear and mitochondrial DNA. At very high H_2O_2 concentrations of 5.0 to 10.0 mM, or 150 to 300 µmol/10^7 cells and above, cell membranes disintegrate, proteins and nucleic acids denature, and necrosis swiftly follows. Cultured cells grown in 20% oxygen are essentially pre-adapted or pre-selected 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. This broad spectrum of cellular oxidant stress responses, depending on the level of oxidant applied and the level of oxygen in the cell culture system, provides for a new paradigm of cellular oxidative stress responses.

Introduction to Oxidative Stress

It has been said that, "a disturbance in the pro-oxidant/anti-oxidant systems in favor of the former may be denoted as an oxidative stress" (1). Oxidative stress can result from increased exposure to oxidants or from decreased protection against oxidants; both problems may even occur simultaneously. This view of oxidative stress as an imbalance between oxidant exposure and oxidant protection is strongly supported by an extensive literature (*e.g.* 1-6). Although oxygen is by no means the only oxidizing agent to which cells or organisms are exposed, it is certainly the most ubiquitous.

Ground-state (or triplet) molecular oxygen is the major form of element number 8 encountered environmentally, and it contains two unpaired electrons. In other words, molecular oxygen is a bi-radical (2,3). A small percentage of the oxygen in our environment actually exists in a singlet state, caused by absorption of electromagnetic energy. The two unpaired electrons in triplet molecular oxygen have parallel spins. Absorption of energy (e.g. from UV light) transiently "flips" one of these spins producing the short-lived, spin-paired, singlet oxygen species. Ground state molecular oxygen is only a moderately active oxidizing agent because the "spin restriction rule" prevents its full reduction by two spin-paired electrons (2,3). Since singlet oxygen can directly accept two spin-paired electrons it is a more powerful oxidizing agent than ground-state oxygen (2,3). Singlet oxygen is thought to be a significant mediator of photo-oxidative stress.

The spin-restriction rule causes most oxygen reduction reactions to proceed one-electron at a time (2,3). This series of one-electron oxygen reduction steps has become known (1-6) as the univalent pathway for oxygen reduction (Scheme 1). In the univalent pathway, oxygen is first reduced to the superoxide anion radical and then to hydrogen peroxide. A third electron reduction (with water elimination) produces the extremely powerful oxidant, hydroxyl radical ($^{\cdot}OH$). This dangerous univalent pathway finally grinds to a halt with the production of water, following the fourth-electron reduction. Ground state oxygen itself is a mild oxidizing agent; whereas singlet oxygen, hydrogen peroxide, and the hydroxyl radical are stronger oxidants. Superoxide is actually a mild reducing agent which can donate an unpaired electron to selected cellular constituents, thus beginning a damaging free radical cascade (5).

Other, related, oxygen-based oxidants are also of biological importance (1-6). These include the chlorinated oxygen products hypochlorous acid and hypochlorite (HOCl and OCl^-) produced by phagocytes as an antibacterial defense, and nitric oxide ($NO^{\cdot}$) used for blood vessel vasodilation; as well as the product of its reaction with superoxide, peroxynitrite ($ONOO^-$). Many of the initial products of cellular oxidation actually act as more powerful propagators of oxidative damage; these include lipid peroxides, oxidized proteins, and oxidized sugars. Finally, living organisms are exposed to many oxidizing environmental agents, foods, medications and drugs. Significant environmental oxidants include ozone (O_3), various oxides of nitrogen (NO_X), numerous products of (industrial) partial combustion, and various pesticides and herbicides (e.g. paraquat). Some food items are direct oxidants produced by cooking, some are strong catalysts of oxidation (e.g. iron and copper), while others still may undergo slow oxidation by oxygen in the body (e.g. various sugars and sulfhydryls). Several medications and drugs are metabolized to form oxidizing agents (e.g. acetaminophen), while others actually catalyze oxygen radical production by mitochondria (e.g. doxorubicin or Adriamycin™).

The oxidative stress inducing agents and conditions discussed above can cause damage to proteins, lipids, carbohydrates, and nucleic acids (1-6). Thus cellular enzymes and structural proteins, membranes, simple and complex sugars, and DNA and RNA are all susceptible to oxidative damage (1-6). Surviving an oxidizing environment is actually one of the greatest challenges faced

by living organisms. The concept that over time we eventually surrender to a form of organic "rust" led to

the free radical theory of aging (7,8).

Antioxidant Defense & Repair Systems

The first-level of cellular responses to oxidative stress is to use antioxidant defense and repair system to minimize the damage that actually occurs, and to remove or repair whatever cellular components do get damaged (1-6, 8-11). Although living organisms may eventually succumb to oxidant-induced aging, levels of oxidatively damaged cellular constituents are actually maintained at very low levels for most of the life span. Minimal damage accumulation is achieved by multiple interacting systems of antioxidant compounds, antioxidant enzymes, damage removal enzymes, and repair enzymes. In addition aerobic organisms maintain very tight control of both oxygen perfusion and cellular/tissue oxygen concentration.

Antioxidant compounds include the well-known vitamins C and E, ubiquinone, uric acid, and many others. Antioxidant compounds are "sacrificed" to oxidation in order to directly protect more important cellular components (1-4,6). Antioxidant enzymes, such as superoxide dismutases, glutathione peroxidases, and quinone reductases, act catalytically to convert oxidants to less reactive species (5,6). The essential cytoplasmic and nuclear proteolytic enzyme, proteasome recognizes and selectively degrades oxidized proteins (6,8,9). Oxidized membrane lipids are recognized and selectively removed by lipases, particularly phospholipase A_2 (6,8,10). Oxidatively modified DNA is subject to removal or excision repair by a wide series of DNA repair enzymes including endonucleases, glycosylases, polymerases, and ligases (6,8,11).

Last, but certainly not least, oxygen concentration is tightly controlled in multicellular organisms, directly limiting the possibility of oxidation. In human beings the respiratory and circulatory systems combine to perfuse tissues with oxygen, and remove carbon dioxide. Nevertheless, oxygen concentration falls from atmospheric levels (20%) in the lungs to only 2-5% in the tissues. In addition the very low K_M of mitochondrial cytochrome oxidase for oxygen (12) assures that most oxygen in cells is actually bound by the cytochrome oxidase complex. Thus a major component of oxidative stress defenses is to keep actual cellular oxygen tension to a minimum. This fact is often overlooked by scientists performing cell culture experiments in which cells are directly exposed to atmospheric oxygen levels. By definition such cells have already been selected or adapted to be able to grow under conditions of very high oxidative stress.

Mitogenic effects of Low Oxidant Concentrations

Exposure of dividing mammalian cells in culture to low concentrations of oxidants actually stimulates

cell growth and division (Scheme 2). This fascinating mitogenic effect is seen, for example, with exposure of fibroblasts to hydrogen peroxide at 3 to 15 µM, or 0.1 to 0.5 µmol/10^7 cells (13-15). Presumably at such low concentrations H_2O_2 does not cause a true oxidative stress. In all probability a level of H_2O_2 in this range actually acts as a signaling agent for mitosis, although the mechanism is unknown. Interestingly, this growth-stimulatory effect of very low H_2O_2 concentrations is also seen with bacteria (16,17) and yeast cells (18).

Temporary Growth-Arrest as a Defense Against Oxidative Stress

Although 3 µM H_2O_2 is mitogenic, 120 to 150 µM hydrogen peroxide, or 2 to 5 µmol/10^7 cells, causes a temporary growth-arrest (Scheme 2) in mammalian fibroblasts (15). This temporary growth-arrest lasts for some 3-6 hours, and appears to be caused by expression of the *gadd*45, *gadd153* (19-21), and *adapt*15 genes (22,23). Many investigators have treated all growth-arrest responses as toxic outcomes of oxidant exposure. I would like to suggest that temporary growth-arrest is actually a defense mechanism. During peroxide-induced temporary growth-arrest, the expression of many housekeeping genes is halted, while expression of a select group of shock or stress genes is induced. This response can be viewed in analogy, as the "Medieval Castle Defense Against Toxicity." When attacked by an invading army the inhabitants of castles in medieval times would raise the draw bridge, lower the portcullis, conserve their resources, and hope that they could outlast their attackers. Similarly, I suggest, proliferative cells exposed to 10^{-6} M H_2O_2 shut-off expression of all but the most essential shock/stress genes, supercoil their DNA to protect it against oxidation, temporarily arrest divisional processes, and conserve resources for future use.

Interestingly, it does not matter which stage of the cell cycle fibroblasts are in, when a hydrogen peroxide stress is applied. At any point in the cell-cycle, application of 120 to 150 µM hydrogen peroxide, or 2 to 5 µmol/10^7 cells, simply stops cell-cycle progression for 3-6 hours (22,23); after which the cells are at least temporarily synchronized. Low µM levels of H_2O_2 are easily metabolized (largely by glutathione peroxidases and catalases) during a 3-6 hour temporary growth-arrest period so that when cells re-enter the growth cycle there is no longer a threat to counter. I propose that temporary growth-arrest is used as an effective counter measure against a wide variety of toxic agents, not just hydrogen peroxide.

Transient Adaptation to Oxidative Stress

Very important early studies on transient adaptation to oxidative stress were performed by Spitz *et al.* (24) and by Laval (25). After 4-6 hours of temporary growth-arrest many cells exposed to 120 to 150 µM hydrogen peroxide, or 2 to 5 µmol/10^7 cells, undergo further changes that can be

characterized as transient adaptation to oxidative stress (Scheme 2). In mammalian fibroblasts we (15,22,23,26-31) and others (24,25) have studied maximal adaptation is seen approximately 18 hours after

initial exposure to hydrogen peroxide; i.e. some 12-14 hours after they exit from temporary growth-arrest. In bacteria such as *E. Coli* and *salmonella* maximal adaptation is seen 20-30 minutes after oxidant exposure (16,17,26), whereas yeast cells require some 45 minutes for maximal adaptation (18,26).

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 transient, lasting some 18 hours in mammalian fibroblasts, 90 minutes in yeast, and only 60 minutes in E. Coli. In our studies we have been especially careful to avoid selecting for pre-existing resistant cells in the population, by repeatedly checking that transiently adapted cells can actually de-adapt.

In both procaryotes and eucaryots, transient adaptation to oxidative stress depends upon transcription and translation. A large number of genes undergo altered expression during the adaptive response. Some genes are up-regulated, some are down-regulated, 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 hours following H_2O_2 exposure, one at 4-8 hours, and one at 8-12 hours. 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 mRNA's. It further appears that message stabilization (for some mRNA's), increased message degradation (for other mRNA's), and altered precursor processing, are all involved in altered translational responses (6,15,18,22,23,26-31).

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 (32) and the soxRS regulon (33). 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. As indicated in the section above, *gadd153*, *gadd45*, and *adapt15* play important roles in inducing temporary growth-arrest, which is a very important early portion of the adaptive response to oxidative stress (15,19-23). The transcription factor, adapt66 (a *mafG* homologue) is probably responsible for inducing the expression of several other adaptive genes (28). 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* (27), and another is *adapt73* which appears to also be homologous to a cardiogenic shock gene called *PigHep3* (29). *Adapt 78* has also been called *DSCR1* (30,31) and,

in addition to its induction during oxidative stress adaptation, now appears to also be involved in Down Syndrome, Parkinson's Disease, and Alzheimer's Disease.

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* (34), c-*jun*, *egr*, and JE (35-37). Similar oncogene induction has also been reported following exposure to *tert*-butyl-hydroperoxide (36,37). The induction of heme oxygenase by many oxidants, including mild peroxide stress, may have a strong protective effect, as proposed by Keyse and Tyrrell (38). 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 (39); interleukin-8 (40); catalase, glutathione peroxidase, and mitochondrial mangano-superoxide dismutase (41); natural killer-enhancing factor-B (42); mitogen-activated protein kinase (43); and gamma-Glutamyl transpeptidase (44). Relatively low levels of nitric oxide have also been shown to induce expression of c-*jun* (45), c-*fos* (45,46), and zif/268 (46). 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 paragraph have not actually been studied in an adaptive cell culture model. Thus, although many of the genes discussed in this paragraph appear to be excellent candidates for involvement in transient adaptation to oxidative stress, their actual importance remains to be tested.

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.* (47). 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.

Permanent Growth-Arrest

If dividing mammalian cells are exposed to higher concentrations of H_2O_2 than those that cause temporary growth-arrest and transient adaptation, they can be forced into a permanently growth-arrested state (Scheme 2). Thus, cells exposed to H_2O_2 concentrations of 250 to 400 µM, or 9 to 14 µmol/10^7 cells, will never divide again (15).

Countless cytotoxicity studies have measured "cell death" by loss of proliferative capacity. These include studies with oxidizing agents, alkylating agents, heavy metals, various forms of radiation, etc. The common assumption of such investigations is that loss of divisional competence equals cell death. It is, indeed, true that many cells exposed to sufficient stress will both stop dividing and die (see next two

sections). It is now clearly incorrect to conclude, however, that all permanently growth-arrested cells will die as a direct consequence of toxicant exposure. In other words, cells may survive an oxidative stress yet be permanently growth-arrested (15,48).

Studies of both cell populations and individual cells have revealed that cultured mammalian cells (normal doubling time of 24-26 hours) can survive for many weeks (at least) following exposure to 100-200µM H_2O_2 without dividing again (15). In the past, studies estimating percent cell viability by growth curves or colony formation measurements alone have completely missed this permanent growth-arrest response. Arrested cells still exclude trypan blue, maintain membrane ionic gradients, utilize oxygen, and make ATP. Interestingly, such permanently growth-arrested cells may make good cellular models for certain aging processes (48). Whether the cessation of proliferation induced by oxidative stress (or other stressful exposures) in some way mimics the loss of divisional competence typical of terminally differentiated cells remains to be seen.

Cell Suicide or Apoptosis

A fraction of cells exposed to higher H_2O_2 concentrations of 0.5 to 1.0 mM, or 15 to 30 µmol/10^7 cells, will enter the apoptotic pathway (Scheme 2). The mechanism of oxidative stress-induced apoptosis appears to involve loss of mitochondrial transmembrane potential (49), release of cytochrome *c* to the cytoplasm (50), l Last, but certainly not least, oxygen concentration is tightly controlled in multicellular organisms, directly limiting the possibility of oxidation. In human beings the respiratory and circulatory systems combine to perfuse tissues with oxygen, and remove carbon dioxide. oss of bcl-2 (51), down-regulation and degradation of mitochondrially encoded mRNA, rRNA, and DNA (52-54), and diminished transcription of the mitochondrial genome (55). Current thinking about toxicant-induced apoptosis suggests that, in multicellular organisms, the repair of severely damaged cells represent 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 (56). Such cells are engulfed by phagocytes which prevent an immune reaction and recycle usable nutrients (49-58).

Certain toxicants, such as staurosporine, can induce widespread apoptosis in fibroblast cell cultures, with greater than 80% cell suicide (53,55). Even higher levels of apoptosis (98% or more) are routinely observed upon withdrawal of IL-2 from *in vitro* cultures of T-lymphocytes (53,55). In contrast, the highest levels of apoptosis we can induce in fibroblasts by H_2O_2 exposure never exceed 30-40% (53). The cause of such disparity is not at all clear but it may suggest either slightly

divergent pathways to apoptosis, or different efficiencies of repair processes for various toxicants. 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 (49) and

down regulation and degradation of mitochondrial polynucleotides (52-54), 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 (57). These observations support a strong involvement of oxidative mechanisms stress in general apoptotic pathways.

Cell Disintegration or Necrosis

At even higher concentrations of hydrogen peroxide, e.g. 5.0 to 10.0 mM, or 150 to 300 µmol/10^7 cells, cells simply disintegrate or become necrotic (Scheme 2). Membrane integrity breaks down at such high oxidant stress levels and all is then lost (15,59). Studies that purport to examine cellular responses to 10.0 mM H_2O_2 in mammalian fibroblasts are really not looking at the responses of cells, but rather at the release of components those cells originally contained. At a high enough level of oxidative stress (e.g. over 10 mM H_2O_2) all mammalian cell cultures will turn into a necrotic "mess" (15). Oxidation induced necrosis may play a significant role in ischemia-reperfussion injures such as heart attacks, strokes, ischemic bowel disease, and macular degeneration. Unfortunately, necrotic cells cause inflammatory responses in surrounding tissues. Such secondary inflammation (also an oxidant stress) may be particularly important in many auto-immune diseases such as rheumatoid arthritis and lupus.

Summary

Oxidative stress causes a very wide spectrum of genetic, metabolic, and cellular responses, most of which are designed to protect cells from damage and deterioration. These include Antioxidant Defenses; Transient Growth Arrest; Direct Repair Systems; Damage Removal, Repair, and Replacement Systems; and Adaptive Responses to Free Radicals and Oxidative Stress. 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 tissues.

Cultured cells grown in 20% oxygen are essentially pre-adapted or pre-selected 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.

Cellular redox state is now well-known as a mediator of various signal-transduction pathways (59). Finally, it now appears that antioxidant compounds, such as vitamin E, may also control the expression of several genes involved in responses to oxidative stress, and regulate key, post-translational, phosphorylation/dephosphorylation steps that regulate signal transduction pathways (60). This raises the

exciting possibility of a very fine tuning of oxidant stress responses, by both positive and negative feedback loops.

Naturally, the full spectrum of responses described in this review can only be seen in proliferating cells such as those from the liver, intestinal lining, skin, *etc*. Nevertheless, even non-proliferating cells from such organs as brain, heart, and skeletal muscle will exhibit altered gene expression and metabolism at tolerated levels of oxidative stress, and are subject to apoptosis or necrosis at higher stress levels. The full spectrum of cellular responses to oxidative stress must, therefore, be considered when applying the knowledge gained in cell-culture studies to human diseases, and aging.

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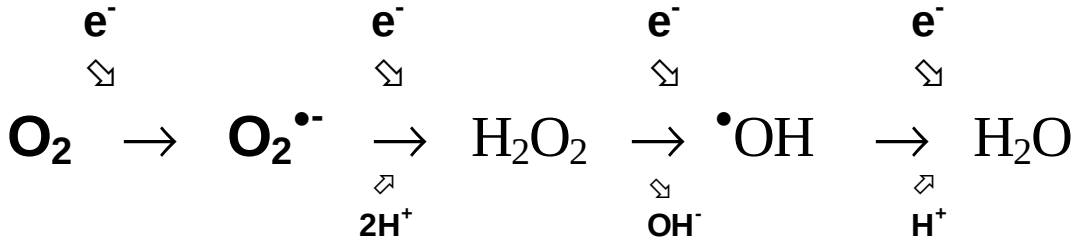
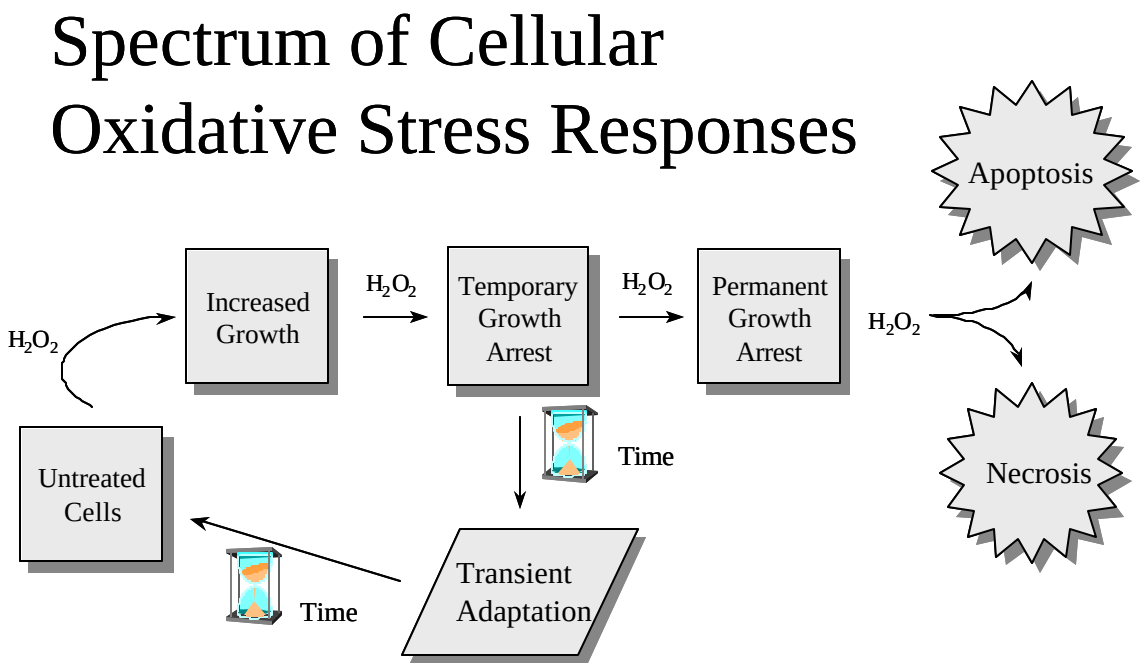
Scheme 1

The Univalent Pathway for Oxygen Reduction

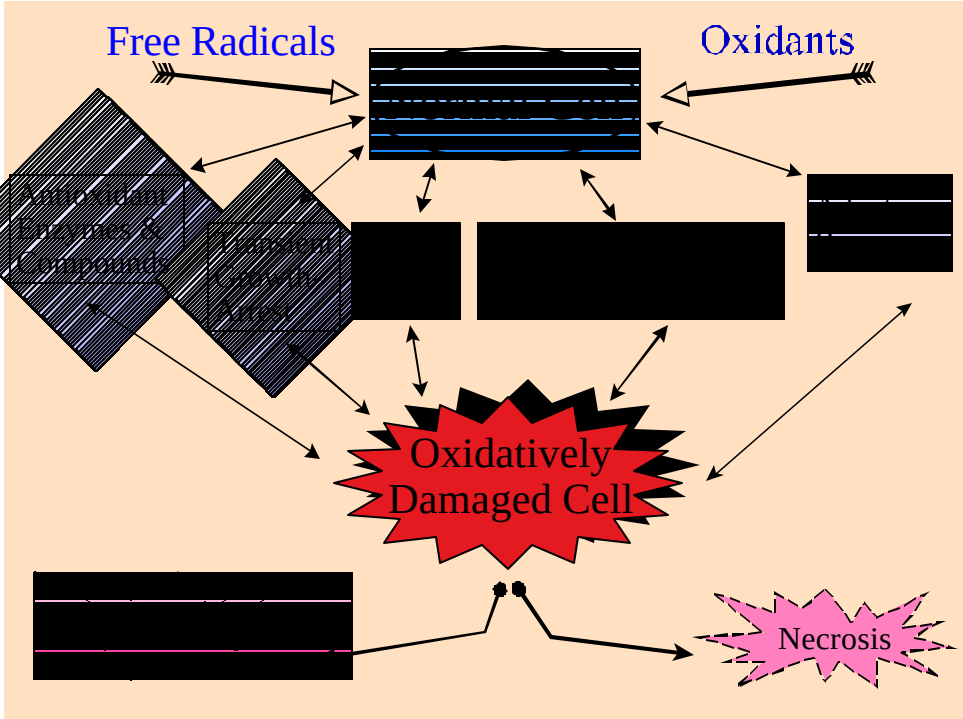


$O_2^{\cdot -}$ = superoxide, H_2O_2 = hydrogen peroxide, $^{\cdot}OH$ = hydroxyl radical

Scheme 2


Spectrum of Cellular
Oxidative Stress Responses
Apoptosis
Increased Growth
H_2O_2
Temporary Growth Arrest
H_2O_2
Permanent Growth Arrest
H_2O_2
H_2O_2
Necrosis
Time
Untreated Cells
Time
Transient Adaptation

Scheme 3



Oxidative Stress, Antioxidant Defenses, and Damage Removal, Repair, and Replacement Systems. The schematic depicts, in cartoon fashion, the conversion (by free radicals or other oxidants) of a normal, mitotic, eucaryotic 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 hours after stress exposure, and lasts for no more than 36 hours. 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.