CHAPTER 1

Introduction to free radicals, inflammation, and recycling

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This introductory chapter will give you information to fill in the gaps and understand the complexities reported in Chapters 3–31.

THEMATIC SUMMARY BOX

At the end of this chapter, students should be able to:

- Show free radical formation
- Show how endogenous and exogenous free radicals stimulate and potentially initiate disease
- Define inflammation and the immune response
- Differentiate between acute and chronic inflammation
- Describe pathways leading to apoptosis, necrosis, cell death, and disease
- Define pathogenesis
- Show how antioxidants scavenge free radicals and participate in recycling pathways
- Describe biomarker measurements using their abbreviations

Historical perspective

In 1993, an International Symposium on *Free Radicals in Diagnostic Medicine: a systems approach to laboratory technology, clinical correlations, and antioxidant therapy* was organized, and in 1994 it was published as volume 366 in *Advances in Experimental Medicine and Biology*.[1] This was the first attempt to coordinate the various laboratory findings from research publications that were divided into subsections on pathophysiology, analysis, organ-specific disorders, systemic involvement, and therapeutic intervention. In 2007, another International Symposium on Free Radicals in Biosystems was conducted.[2] These two conferences set the standard for the present textbook, which is an extension of those meetings in the application and understanding of free radical (FR) methods and protocols, and is once

Oxidative Stress and Antioxidant Protection: The Science of Free Radical Biology and Disease, First Edition. Edited by Donald Armstrong and Robert D. Stratton.

again timely 20 years later. The author of this chapter has multiple books in the field.

In 1990, a new series of books covering laboratory techniques in *Methods in Molecular Biology* (MiMB) was initiated and later became Advanced Protocols 1–3 including separate volumes on lipidomics and nanotechnology that contain specific sections on oxidative stress and antioxidants. This was followed in 2000 by a new series devoted to clinical studies (*Oxidative Stress in Applied Basic Research and Clinical Practice*), which increased our oxidative stress library database to over 1300 research collaborators from 34 countries and 20 states in the United States.

These books have been a major effort to cover subject areas in advanced detail, which are germane to background information that supports perturbations of lipids and proteins in our concept of oxidative stress. In addition, platform technology on metabolomics and transcriptomics using high-pressure liquid chromatography (HPLC) and mass spectroscopy (MS) can be read in the literature by the student separately and integrated with the state-of-the-art coverage. The result of these activities is the basis for the present textbook, which illustrates the concept of *educational research* in stress-induced disease reactions.

Oxidative stress concept

The concept of oxidative stress in disease means an environmental stimulus is able to create an FR by random chance. The number of "hits" per day is estimated at 100,000 coming from the mitochondria as molecular oxygen moves through the electron respiratory chain and from environmental radiation. FRs are characterized by loss of an electron-making species highly reactive to other biochemicals resulting in cellular damage (Figure 1.1). We think of FRs as tipping the balance toward disease, so that as FRs increase along the up slope as a function of progressive disease, antioxidants (AOXs) decrease along the down slope as they are being consumed due to oxidative stress, so that homeostasis tips toward disease. Supplementation with AOX nutraceuticals leads to protection and eventual homeostasis when the two processes become equal, and no disease is clinically evident by conventional physiological testing in the patient populations.

The most damaging FRs are the hydroxyl ($HO^{\bullet}$) and hydrogen radicals produced during ionizing radiation or environmental toxicology reactions. Superoxide FRs

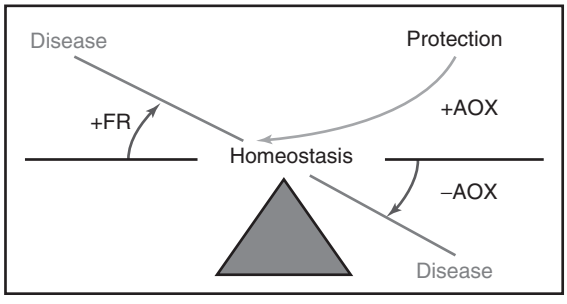

Figure 1.1 Homeostasis is a balance between levels of free radicals (FR) and antioxidants (AOX).

($O_2^{\bullet -}$) are produced in mitochondrial electron transport reactions. Superoxide dismutase rapidly forms hydrogen peroxide (H_2O_2) from $O_2^{\bullet -}$. Reduced free iron Fe^{2+} and copper Cu^{1+} participate in Fenton reactions with H_2O_2 in the millisecond range to produce hydroxyl radicals and hydroxide anions, oxidizing the metals to Fe^{3+} and Cu^{2+}. Oxidized free iron or copper can then oxidize H_2O_2 to form hydroperoxyl ($HOO^{\bullet}$) radicals and protons (H^+), reducing the metals to Fe^{2+} and Cu^{1+}, so that new peroxidation reactions can occur in a cyclical manner. Thiol ($SH^{\bullet}$) and lipidperoxide radicals ($L^{\bullet}/LO^{\bullet}/LOO^{\bullet}$) are other radicals that cause FR damage to cellular components.

Hydrogen peroxide also interacts at Fe^{2+} metal-binding sites on heme-containing enzymes to generate the reactive hydroxyl radical. The hydroxyl radical damage to proteins has been shown to produce covalently bound protein aggregates, and when disulfide bridges are causing the aggregation, cross-linked protein adducts are formed. These reactive oxygen radicals modify amino acids at metal-binding sites and facilitate proteolytic attack. Other FR damage includes DNA strand scission and lipid hydroperoxidation (LHP).

Polyunsaturated fatty acids (PUFA) contain multiple carbon–carbon double bonds. These fatty acids provide mobility and fluidity to the plasma membrane, properties which are known to be essential for the proper function of biological membranes. The process of *lipid peroxidation (LHP)* is a step-wise process with the removal of an electron at the initiation step and at subsequent propagation reactions. Iron salts and other iron complexes help initiate the process by forming alkoxy or peroxy radicals upon reaction with oxygen species.

In general, there are three damaging consequences of LHP within the plasma membrane *in vivo*. The first consequence is a decrease in membrane fluidity. Saturated fatty acids are structurally more rigid than the flexible PUFA. The second consequence of LHP is an increase in the "leakiness" of the membrane pores to substances, which normally do not pass through the membrane. Finally, membrane-bound proteins are damaged by propagation reactions of LHP. Hydroxyl radicals remove a hydrogen atom from methylene groups in a PUFA resulting in a PUFA lipid radical. The lipid radical then reacts with "normal" oxygen ("triplet" O_2) to form a lipid peroxyl radical, which then reacts with another PUFA to form another lipid radical. This propagates the production of reactive oxygen species (ROS) in what is called the peroxidative chain reaction (Figure 1.2).

Reactive nitrogen species (RNS) can also participate in nitrosative oxidative damage. Nitric oxide synthase is the enzyme that drives this reaction. FRs can attack proteins, lipids, carbohydrates, and nucleic acids causing extracellular matrix, cellular, and subcellular damage. Nitrous oxide + superoxide → peroxynitrite ($ONOO^-$), which yields the $NO_2^{\bullet}$ reactive oxidant.

Oxidative stress is highest in the plasma membrane, mitochondria, nucleus, golgi, and lysosomes. AOXs represent about 50% of total Internet citations and cover anti-cancer, anti-inflammatory, and anti-proliferation. FRs can also act in signaling and function as messenger agents.

Inflammation is most often the initial step in a disease process followed by an immune response, but the immune response may occur at nearly the same time. With time, various molecular dysfunctions develop into a chronic disease such as documented in diabetes, cardiovascular disease, organ failure, and cancer.

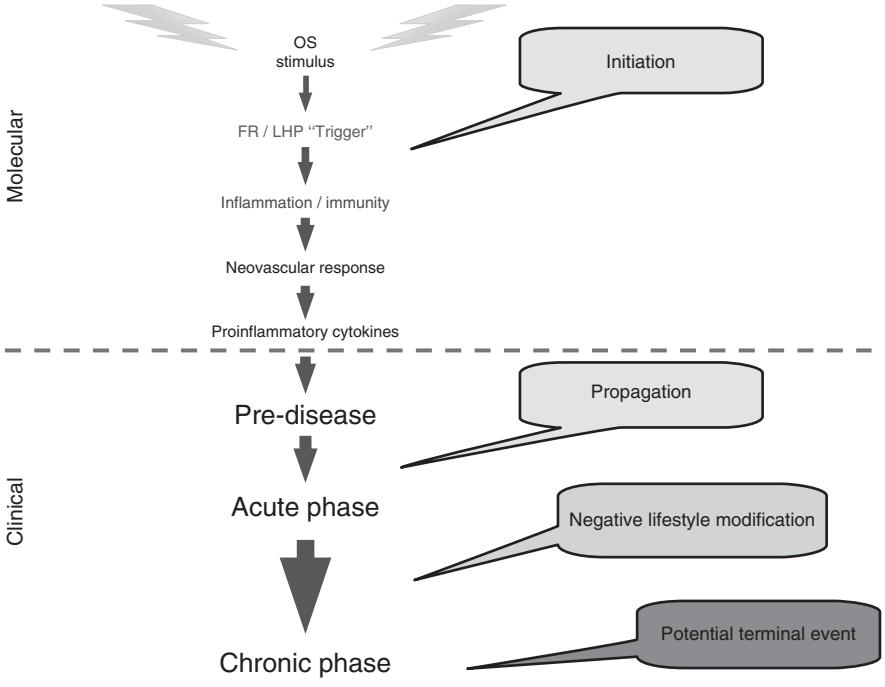

Figure 1.2 Oxidative stress starts a cascade that can lead to chronic disease if not modified by corrective actions.

The first step is called *initiation,* followed by *propagation*, *termination*, and/or *protection.* The AOX pathways are generated by endogenous (internally synthesized) or exogenous sources that come from dietary preferences and lifestyle controlling modifications.

The pathway originates from stimuli that trigger radical formation. These molecular events lead to inflammation and immune responses, provoke neovascularization, and upregulate proinflammatory cytokines. Protective scavenger AOXs are divided into small water-soluble molecules (vitamin C, glutathione (GSH)), lipid-soluble molecules (vitamin E, lipoic acid, carotenoids, and coenzyme Q_{10} (CoQ_{10})), and larger enzyme molecules that need to be synthesized internally (superoxide dismutase, catalase, and GSH peroxidase). These detoxify aqueous and lipid-soluble peroxides. Preventive AOXs bind to essential proteins (albumin, metallothionein, transferrin, ceruloplasmin, myoglobin, and ferritin).

These factors can lead to an "imbalance" – a lack of AOXs because of their underproduction, misdistribution, or environmental stressor depletion. In the clinical realm, these can lead to pre, acute, and chronic disease and may advance to a potentially terminal event.

Biomarkers of oxidative stress can be analyzed in cells; tissues; blood; urine; CSF; synovial fluid; saliva; tears; and many other substances such as botanical nutraceuticals, marine algae, and food samples. Key markers used in most scientific publications are shown by the following oxidative stress metrics (Table 1.1).

Table 1.1 Biomarkers for oxidative stress.

Organelle	Biomarker activity
Nucleus	8-Hydroxy deoxyguanosine
Mitochondria	Catalase, Cu/Zn-SOD, Mn-SOD
Endoplasmic reticulum and golgi	PEG-SOD, F2-isoprostanes, HNE
Plasma	TBARS, CUPRAC, 8-iso-PGF(2α), LHP
Total cellular constituents	Cytokines, chaperones, telomeres

Cytokines are immunomodulatory agents that act as intracellular chemical mediators. They activate antigens and carry signals to adjacent cells of the immune system, thus magnifying the response to disease. Chaperones are functionally related groups of proteins synthesized in the endoplasmic reticulum. They are cellular machines that assist in protein folding and protect against degradation. Under physiological stress, cells respond to an increase by less than 5°C of temperature to produce heat shock proteins, which participate in anoxia, inflammation, and oxidant injury. They can be analyzed by electron microscopy.

Oxidative stress plays a *major* role in many human diseases and may well become the salient feature in most diseases. To date, involvement of oxidative stress has been confirmed in over *100* disorders. Oxidative stress has been previously linked to a plethora of changes induced during aging as well as in specific diseases such as obesity, diabetes, cancer, cardiovascular disease, stroke, neurodegenerative disease, trauma, hypoxia, psychological behavior, pain, chronic fatigue, fibromyalgia, pulmonary disease, hepatic disease, renal disease, gastrointestinal disease, macular degeneration of the eye, disorders of noise-induced hearing loss, fertility, menopause, osteoporosis, endocrine disorders, skin disease, musculoskeletal disorders, bone marrow abnormalities, oral health, nutrition, environmental health, and complications following extended space travel. Genetics may also play a role in the overall pathology of oxidative stress. Many of these topics have extensive publications in peer-reviewed scientific and clinical journals, corroborating the role of oxidative stress. Therefore, oxidative stress should be considered a *primary* cause of most diseases, or at least the result of several compounded processes that require more data and are currently under investigation. The student should consult the Internet, PubMed, Citation Index, or ISI Web of Science to study the many oxidative stress-related activities present in tissues and organs measured with new appropriate biomarkers.

Free radicals

Oxidation reactions cause the formation of a variety of FRs, which are unstable substances, that can initiate chain reactions in *microseconds*, leading to disease and programmed apoptotic cell death. Cells may recover or may undergo apoptotic autophagy or uncontrolled necrosis. Necrosis is when the tissue cannot regulate the influx of fluids and the loss of electrolytes, most notably in mitochondria and is associated with extensive damage resulting in an inflammatory response.

Apoptosis is by definition a phenomenon of programmed cell death under normal homeostasis control, and consequently no inflammatory response is observed. The cell shows membrane blebbing, shrinkage in size, nuclear condensation, DNA chromatin fragmentation, aggregation of chromatin, nuclear condensation, and partition of cytoplasm into membrane-bound vesicles, which contain ribosomes and nuclear material. These are phagocytized by macrophages, but there is no inflammatory response. Chronic apoptosis may cause widespread atrophy.

Free radicals may occur from a specific stimulus such as ultraviolet radiation, multiple environmental factors, poor nutrition, or sedentary lifestyle. Oxidative stress can stimulate neutrophils in the blood to ingest pathogens, but these are replaced on a daily basis by younger cells. FRs can attack proteins, lipids, carbohydrates, and nucleic acids causing cellular and subcellular damage to cells by ROS or RNS (Figure 1.3).

Note that the amount of oxidized protein is proposed as the tipping point in the FR and AOX balance scheme. Oxidized proteins activate the caspase enzyme cascade in the proteasome, form intracellular aggregates, and are predominately nonrepairable because cross-links limit repair mechanisms so that recycling of amino acids for continuous protein synthesis is diminished.

The master AOX for recycling and FR inactivation is reduced GSH, but lutein and phenols with hydroxyl groups can readily take up unpaired electrons together with ascorbate; α-tocopherol/tocotrienol; and enzymes such as superoxide dismutase, catalase, GSH peroxidase, GSH reductase, and CoQ_{10}.

Inflammatory pathways

Inflammation facilitates healing from noxious or foreign stimuli. The initiating event is tissue damage. It involves the formation of nuclear factor kappa B (NFκB) and systemic cytokine by-products such as TNF-α and prostaglandin E2-α. Biomarkers are thiobarbituric acid reactive substances (TBARS), derivatives of reactive oxygen metabolites (dROMS), oxygen radical absorbance capacity (ORAC), hydroxyl radical antioxidant capacity (HORAC), arachidonic acid, thromboxane, lipopolysaccharides, and trolox equivalent antioxidant capacity (TEAC)). Many biomarkers degrade over

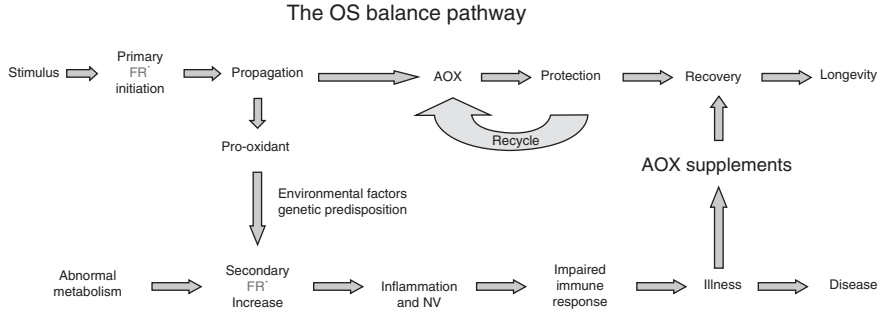

Figure 1.3 Free radicals (FR) are key to initiation and propagation of the paths that lead to disease. Antioxidants (AOX) are key to protection.

time, so it is advised to use fresh or freshly frozen samples. For example, in type 2 diabetic patients, oxidative stress is closely associated with chronic inflammation by upregulating key vascular ROS- and RNS-producing enzymes and the corresponding endogenous AOXs. Heme oxygenases (HO-1) utilize NADPH and oxygen to rupture the heme moiety, causing modulation of cellular bioenergetics and leading to apoptosis and inflammation. These are my interpretations of oxidative stress and AOX events in disease to gain a mechanistic summary and thus an approach to therapy.

Mitochondria

Molecular oxygen diffuses into the mitochondrial inner membrane where ROS sources are actively produced through the electron transport chain and nitric oxide synthase reactions. Cytochromes are present in mitochondria and transfer electrons along the respiratory chain that involves oxidation and reduction of iron. CoQ_{10} is a naturally occurring AOX and a prominent component of mitochondrial electron transport chain. CoQ_{10} is recognized as an obligatory cofactor for the function of thermogenesis uncoupling proteins and a modulator of the mitochondrial transition pore. It was also observed that CoQ_{10} is part of an endogenous AOX defense that increases SOD2 and GSH peroxidase. In disease-prone cells, mitochondrial superoxide is exported to adjacent cells, triggering lipid peroxidation, propagation reactions, and inflammation.

Educational redox

The following list of information on products for redox therapy as an experimental therapy is based on results-oriented data. The following list has contact information from prominent companies specializing in pro-oxidant and AOX agents in cells. These also cover alternative and holistic medicine.

1. Life Extension Foundation, 5th edition, 2013 (www.lifeextension.com/track)
2. Integrative Therapeutics, Inc (www.integrativepro.com)
3. (www.naturalmedicines.therapeuticresearch.com)
4. Advanced Bionutritionals (www.advancedbionutritionals.com)
5. Oxford Biomedical Research (http://www.oxfordbiomed.com)
6. OXIS Research International (www.oxisresearch.com)
7. Cayman Chemical Co. (www.caymanchem.com)
8. ALPCO Diagnostics (www.alpko.com)
9. The Japan Institute for the Control of Aging, Nikken SEIL Corp. (www .biotech@jaica.com), Genox is the USA distributor.
10. ALEXIS Biochemicals (www.alexis-biochemicals.com)
11. INOVA Diagnostics (www.inovadx.com)
12. Molecular Probes by Life Technologies (www.lifetechnologies.com)
13. PROBIOX SA, Belgium (www.probiox.com)

14 The National Center for Comparative Alternative Medicine (nccam.nih.gov) is an investigator-initiated project on advanced research covering complementary and alternative medicine.

Great variability in the activities of AOXs is present in over-the-counter nutraceuticals. Disclaimers for food products that are not under FDA regulation are treated as a food, not as a pharmaceutical. The caveat that must be put on the label is "These statements have not been evaluated by the FDA and the product is not intended to diagnose, treat, cure, or prevent any disease." Therefore, be careful with sources of AOX treatments.

Stay current with new research initiatives. The student is also encouraged to keep a record of these applications. In searching the Internet, the student should key in on *MiMB* and *Oxidative Stress in Applied Basic Research and Clinical Practice*, published by Humana Press, a brand of Springer and part of Springer Science + Business Media.

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