

## Perspectives in Pharmacology

# Oxidative Shielding or Oxidative Stress?

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### ABSTRACT

In this review I report evidence that the mainstream field of oxidative damage biology has been running fast in the wrong direction for more than 50 years. Reactive oxygen species (ROS) and chronic oxidative changes in membrane lipids and proteins found in many chronic diseases are not the result of accidental damage. Instead, these changes are the result of a highly evolved, stereotyped, and protein-catalyzed “oxidative shielding” response that all eukaryotes adopt when placed in a chemically or microbially hostile environment. The machinery of oxidative shielding evolved from pathways of innate immunity designed to protect the cell from attack and limit the spread of infection. Both oxidative and reductive stress trigger oxidative shielding. In the cases in which it has been studied explicitly,

functional and metabolic defects occur in the cell before the increase in ROS and oxidative changes. ROS are the response to disease, not the cause. Therefore, it is not the oxidative changes that should be targeted for therapy, but rather the metabolic conditions that create them. This fresh perspective is relevant to diseases that range from autism, type 1 diabetes, type 2 diabetes, cancer, heart disease, schizophrenia, Parkinson’s disease, and Alzheimer disease. Research efforts need to be redirected. Oxidative shielding is protective and is a misguided target for therapy. Identification of the causal chemistry and environmental factors that trigger innate immunity and metabolic memory that initiate and sustain oxidative shielding is paramount for human health.

*Nothing in biology makes sense except in the light of evolution.*

—Theodosius Dobzhansky (Dobzhansky, 1973)

### Introduction

An alternative title for this review might be, “Oxidative stress or oxidative shielding: can 50 years of research be wrong?” To understand why cells might choose to defend themselves from harm by intentionally making reactive oxygen species (ROS), such as superoxide and hydrogen peroxide, and by stiffening the cell membrane to make it less permeable and less vulnerable to attack, we need to start at the beginnings of life on our planet. The great evolutionary pulses of metabolic and structural innovation of life on Earth

can be correlated with changes in environmental oxygen (Holland, 2006). In the beginning, all life on Earth was anaerobic and oxygen was toxic. The first cells to emerge in the Precambrian seas were anaerobic bacteria that made ATP by anoxygenic photosynthesis. This life chemistry dates to approximately 3.5 billion years ago (GYA) (Cavalier-Smith, 2006). Most of the pathways of intermediary metabolism that we know today were developed during this anaerobic epoch of life’s history on Earth. Isoprenyl and ubiquinol synthesis, fatty acid oxidation and synthesis, iron-sulfur cluster synthesis, glycolysis, carotenoid synthesis, the pentose phosphate pathway, the glyoxylate cycle, pyruvate dehydrogenase, cobalamin synthesis, heme synthesis, cytochromes, glutathione metabolism, electron transport, chemiosmotic proton-coupling for ATP synthesis, and both reductive and oxidative (reverse and forward) Krebs cycles all were present in the oldest bacteria known, the green sulfur bacteria (Tang and Blankenship, 2010). These and other bacterial primary producers fixed carbon from CO<sub>2</sub>, fertilized the seas with organic molecules that later became food, and created the genetic tool

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**ABBREVIATIONS:** ROS, reactive oxygen species; GYA, giga (10<sup>9</sup>) years ago; Torr, millimeters of mercury pressure (mm Hg); GSH, glutathione; GSSG, GSH disulfide; CI, confidence interval; ER, endoplasmic reticulum; pO<sub>2</sub>, oxygen partial pressure.

kit for intermediary metabolism on which all later life was based.

Oxygen came late on the scene. Beginning approximately 2.4 billion years ago, cyanobacteria started producing oxygen from photosynthesis in sufficient quantities to ventilate shallow seas and raise atmospheric oxygen levels to approximately 2 to 4% (Rasmussen et al., 2008; Hohmann-Marriott and Blankenship, 2011). This value of 2 to 4% oxygen [0.02–0.04 atm; 2–4 kilopascals; 15–30 Torr (mm Hg partial pressure) assuming an atmospheric pressure of 760 Torr at sea level] corresponds to approximately 20 to 40  $\mu\text{M}$  dissolved oxygen in shallow, well mixed Precambrian seas. This is very close to the partial pressure of oxygen of most mammalian tissues at the end of capillary beds today and is also very close to the saturation midpoint of hemoglobin A of 28 Torr (Winslow, 2007). The first single-celled eukaryotes with mitochondria may have emerged as early as 1.7 to 1.1 GYA (Knoll et al., 2006; Brinkmann and Philippe, 2007). The saturation midpoint of modern mitochondria for oxygen in state 4 respiration is 0.2 Torr (0.025 kilopascals) or approximately 0.3  $\mu\text{M}$  (Gnaiger et al., 1995). This creates a natural 150-fold oxygen gradient from ambient concentrations of 30 Torr outside the cell to 0.2 Torr at the site of oxygen consumption by the enzyme cytochrome *c* oxidase in each mitochondrion. These ancestral plant and animal cells were dispersed by the winds and tides as plankton, finding chemically unique niches, specializing, and sharing the Precambrian seas with bacteria and viruses. This rich ecology coevolved for a billion years before multicellularity took hold and expanded during the Cambrian approximately 0.54 GYA. The ability of cells to use oxygen to make ATP today, to detoxify it, and to release it as ROS for purposes of cell signaling and defense were developed during this pivotal Precambrian epoch. The free radical theory of aging was proposed by Harman (1956).

*Self-defense is Nature's oldest law.*

—John Dryden, “Absalom and Achitophel,” (1681)

## Mitochondria, Innate Immunity, and Cellular Defense

In this review I present evidence that one of the most ancient functions of mitochondria was cellular defense. Today we call this metabolic response to cellular attack or injury “innate immunity.” Mitochondria play a central role in innate immunity today (West et al., 2011). This is the direct result of the role that bacterial ancestors or mitochondria played in the Earth's early oceans. The complex ecology of the Precambrian seas was replete with viruses and predatory intracellular bacteria (Wichels et al., 1998). No cells without effective defenses survived. The biochemical signature of an attack is a metabolic “steal” or diversion of electrons and resources such as nitrogen, phosphorus, iron, and copper. When limiting cellular resources are used by predators and parasites, those resources are not available to the host cell. Mitochondria are uniquely equipped to detect and respond to this metabolic steal. When the local chemistry of the cell provides nutrients and resources in concentrations that are matched to mitochondrial metabolism, mitochondria will create a normal oxygen gradient of approximately 30 outside the

cell to 0.2 Torr in the mitochondrial matrix (Gnaiger et al., 1995) (see above). When cellular resources are consumed by a parasite, a “metabolic mismatch” is produced.

Mitochondria have a proteome of approximately 1500 proteins (Pagliarini et al., 2008). Nearly 1000 of these proteins have catalytic functions in cell metabolism, such as citrate synthase or malate dehydrogenase. Under normal physiologic conditions, the concentrations of thousands of nutrients and metabolic substrates in mitochondria are closely governed by the collective kinetic constants ( $K_m$ ,  $K_{cat}$ ,  $V_{max}$ , Hill coefficient, etc.) of all the enzymes responsible for transforming those metabolites. This has recently been computationally modeled in the Recon 1 and BiGG reconstructions of cell and organ metabolism (Schellenberger et al., 2010; Rolfsson et al., 2011). Only the primary structure of an enzyme is genetically determined. The activity of an enzyme at any instant in time is determined by ambient metabolic conditions. For example, the  $K_m$  of citrate synthase for oxaloacetate is approximately 2  $\mu\text{M}$ , but the enzyme is allosterically inhibited by ATP, NADH, acetyl-CoA, palmitoyl-CoA, and the product citric acid, so the rate of converting oxaloacetate to citrate is changing minute to minute according to the condition of the cell (Shepherd and Garland, 1969). When the concentrations of substrates are perturbed by viral or microbial infection, disease, toxin, or nutritional excess, mitochondria sense this as a metabolic mismatch between the optimum concentration of those metabolites for a given tissue and the actual concentration.

This metabolic mismatch diverts electron flow away from mitochondria in the cell and decreases intramitochondrial electron flow, and mitochondrial oxygen consumption falls. When mitochondrial oxygen consumption (extraction) falls and the cell is still surrounded by 30 Torr (2–4%) oxygen supplied by capillaries, the concentration of oxygen in the cell rises sharply. When cellular oxygen rises, the redox of the cell rises, and the chemistry of polymer assembly (DNA, RNA, lipid, protein, and carbohydrate synthesis) is ultimately stopped because the NADPH/NADP<sup>+</sup> ratio falls as the change in Gibbs chemical free energy of synthetic reactions becomes less negative (more positive changes in Gibbs chemical free energy are thermodynamically less favorable). Under these more oxidizing conditions, electrons are no longer available for carbon-carbon bond formation to build biomass for viral or intracellular bacterial replication. Electrons are instead abstracted by the rising tide of intracellular oxygen to make superoxide, other ROS, and reactive nitrogen species and form bonds between free thiols in amino acids, such as cysteine, and peptides, such as glutathione, to make disulfides, such as cystine and the glutathione disulfide (GSSG). The rising tide of intracellular oxygen also oxidizes iron-sulfur clusters and redox-responsive sites in many proteins, inactivating proteins for macromolecular synthesis and activating proteins that shield the cell membrane from further attack. These include lipoxygenases and NF- $\kappa$ B (Serezani et al., 2011), NADPH oxidases (Jiang et al., 2011), redox-sensitive signaling systems in innate immunity such as the purinergic receptors (Hillmann et al., 2009), and transcriptional regulators such as Keap1/NRF2, and sirtuin-FOXO (Speciale et al., 2011). **The net result of oxidative shielding in innate immunity is to limit the replication and prevent the exit of the invading pathogen.**

## Oxidative Shielding

Oxidative shielding is a stereotyped response to cellular injury or attack. To better understand the fundamental differences between the oxidative stress and the oxidative shielding perspectives it is helpful to ask and answer a few questions from the viewpoint of these two different schools of thought. This approach is similar to Galileo's dialog (Galileo, 2001) between adherents of Ptolemaic and Copernican systems as a method of showcasing their relative strengths and weaknesses.

**What Triggers the Production of Superoxide, Hydrogen Peroxide, and Other ROS?** Hostile, damaging, or unhealthy conditions surrounding the cell trigger the production of superoxide, hydrogen peroxide, and other ROS. Both stress and shielding schools agree with this answer.

**Where Do the ROS Come From?** ROS come from mitochondria and specialized enzyme systems in the cell. Both stress and shielding schools agree with this answer.

**What Is the Function or Purpose of ROS?** The shielding school holds that the function of ROS is, first, to protect the cell if possible, both as signaling molecules and by physically decreasing the cellular uptake, release, and exchange of potentially toxic pathogens or chemicals from and with the environment; and second, to actively kill the cell by apoptosis or necrosis when the local environmental conditions threaten to spread to neighboring cells and jeopardize the survival of host. ROS are an effect of disease, not the prime cause. In the shielding school, the organism is considered the ultimate unit of Darwinian selection. The fitness of an individual, in terms of its ability to reproduce, can be substantially increased by rapidly cutting off resources, walling off, or actively killing damaged or infected cells in a part of the body to save the whole.

The stress school holds that the function of ROS is to cause cell damage and disease.

**What Is the Target of Effective Therapy in Diseases Associated with Increased ROS and ROS-Related Damage?** The shielding school holds that because the prime cause of disease can ultimately be traced back to toxic exposure, microbial pathogen, unhealthy nutritional practices, nutrient loading, or unhealthy patterns of exercise and activity therapy should be directed at eliminating these causal factors. ROS production will naturally fall back to normal levels when physiologic balance is restored.

The stress school holds that because ROS are the prime cause of disease therapy should be directed at eliminating or normalizing ROS and ROS-related cell damage.

## Randomized Clinical Trials

The scientific literature is rife with cell culture and animal experiments showing apparent benefits of antioxidant therapy and opinion papers that advocate antioxidants for treating everything from diabetes to cancer and Alzheimer's disease. However, the gold standard of proof in medicine is the randomized clinical trial. When antioxidants are put to the test in randomized clinical trials they generally fail or, worse, show evidence of unexpected harm. For example, in a meta-analysis of nine clinical trials that evaluated the benefit of treating type 2 diabetes with antioxidants such as  $\alpha$ -tocopherol (vitamin E) there was no benefit (Suksomboon et al.,

2011). Like many purified antioxidant vitamins, vitamin E is a two-edged sword. The reasons for this are not entirely clear, but may relate to the fact that therapeutic dosing of purified micronutrients and antioxidants intervenes in regulatory pathways that produce biochemical symptoms associated with cell defense, but are not the actual cause of disease. Vitamin E supplementation, alone or in combination with  $\beta$ -carotene, was shown to increase the risk of lung cancer in smokers (The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group, 1994). Vitamin C supplementation was found to double the risk of cancer death in nonobese women [relative risk (RR) = 2.0; 95% CI = 1.12–3.58], while having no effect in obese women (Lin et al., 2009). The SELECT clinical trial of vitamin E and selenium was terminated early because of an apparent increase in the risk of new onset diabetes in the selenium group and a 1.6-fold increased risk in prostate cancer in the vitamin E group (Lippman et al., 2009; Klein et al., 2011). If ROS are at the heart of cancer, diabetes, and heart disease, why are antioxidants so ineffective at preventing or treating these diseases?

Other data that may prove helpful in weighing the merits of the schools of oxidative shielding versus oxidative stress are the results of clinical trials in which a therapy recommended by the shielding school, for example, diet and exercise (which is known to stimulate ROS; Niess and Simon, 2007), is directly compared with conventional medical intervention. When this is done in type 2 diabetes and its prodromal metabolic syndrome, diet and exercise are categorically superior to the best drug intervention. A recent meta-analysis of 13 clinical trials involving 3907 subjects found that the odds ratio for disease improvement with diet and exercise was 3.8 (95% CI = 2.5–5.9), but the odds ratios for disease improvement with drug treatment was 1.6 (95% CI = 1.0–2.5) (Dunkley et al., 2012). Diet and exercise can actually cure early type 2 diabetes while simultaneously reducing the risk of heart disease. In contrast, common drug interventions such as the thiazolidinedione insulin-sensitizing drug rosiglitazone will decrease diabetes, but increase the risk of heart failure (DREAM Trial Investigators et al., 2008).

## Evolutionary Conservation of the Oxidative Shielding Response

The stereotyped oxidative shielding response to danger and metabolic mismatch can be identified in all aerobic forms of life on Earth. Even bacteria have it. For example, *Escherichia coli* rapidly generates superoxide and hydrogen peroxide in a manner reminiscent of mitochondria, by partially reducing oxygen at the site of NADH dehydrogenases and quinone acceptor sites along the inner membrane (Cabiscol et al., 2000). The magnitude of the response is regulated by nutrient availability, environmental toxin, and infection exposure (González-Flecha and Demple, 1997). It is noteworthy that ROS production by bacteria is highest under conditions of nutrient loading, similar to increased ROS production under nutrient loading in diabetes. Inhibitors of NADH dehydrogenase activity, such as the pesticides paraquat or rotenone, produce a rapid increase in ROS and mutation rates in the stressed bacteria (Cabiscol et al., 2000). Antioxidant defenses in aerobic bacteria are coordinately up-regulated by endogenously produced ROS by redox-reactive thiols on cys-

teines of peroxide-responsive OxyR and superoxide-sensitive SoxR transcription factors.

Plant cell ROS production leads to cross-linking of tyrosine-rich proteins in the cell wall (Bradley et al., 1992). Animal cells use many mechanisms including the use of another tyrosine-rich protein, melanin, and the production of collagen scar tissue to wall off the chronically disturbed or injured collection of cells. The initiating rise in intracellular oxygen that is caused by the failure of mitochondria to reduce oxygen to water is the hallmark of a metabolic “fever” or mismatch.

Why is it important to make the distinction between oxidative shielding and oxidative damage? When an oxidative shielding response is beneficial to the cell or the organism, then antioxidant treatments designed to block or reverse it will have two effects: 1) there will be no effect on the primary cause of the cellular toxicity, e.g., the viral infection, toxic exposure, or metabolic mismatch causing the cellular oxidative response, and 2) chronic treatment may ultimately prove harmful because it inhibits the highly evolved protective and hormetic functions of protein-catalyzed oxidative shielding.

### Apoptosis, Cellular Altruism, and the Seductive Clarity of Cell Culture

Cell culture experiments have proved to be highly successful over the years in answering genetic questions. However, cell culture experiments have not been as reliable and are often misleading, in answering metabolic questions when the experiment is aimed at answering a question about multicellular development, organ function, or a whole animal phenotype. This happens because of four major differences between the metabolic conditions of cell culture and tissues. These can be briefly stated as the apoptosis, hyperoxia, cycling cell NADPH, and multicellularity problems.

### Apoptosis: Bad for Cells in Culture, Good for the Species

Mitochondria are the principal regulators of apoptosis (Karbowski, 2010). This process lies at the heart of the developmental program of plants and animals that permits embryos to grow and remove cells that are no longer needed. Another essential function of apoptosis is the physical containment of injury or infection. Cells that become infected by viruses, or other microbial pathogens, initiate the program of apoptotic cell death to prevent the spread of infection. Many viruses and other microbes devote substantial genetic resources to thwart the infected cell's effort to commit suicide (Galluzzi et al., 2008). Evolution has preserved and refined the apoptotic program because it confers an increased fitness to the plant or animal during development and under attack. This can be seen as a form of cellular altruism without intent, in which the death and removal of a few infected, injured, or obsolete cells increases the likelihood of survival of the organism. Protein-catalyzed ROS production and membrane and protein oxidation events precede the commitment to apoptosis in most cell types. These genetic and metabolic pathways have been selected over evolutionary time to increase the fitness of the organism. Ultimately, the oxidative shielding response confers evolutionary ad-

vantage for the organism. This advantage cannot be seen in cell culture because the death of cells in culture occurs without reference to the survival of the whole organism.

### Hyperoxia: The Uniquely Oxidizing Environment of the Culture Dish

Cells in culture are typically grown under ambient oxygen tensions of approximately 100 Torr that result from diffusion from a 21% oxygen atmosphere at sea level. They are not usually grown at the 2 to 4% oxygen (15–30 Torr) that is normal in tissues. Because all of the proteins involved in antioxidant defense evolved under physiologic conditions of 15 to 30 Torr oxygen, they typically have  $K_m$  values for oxygen in the 15- to 30-Torr range. Cell culture hyperoxia in the 30- to 100-Torr range will naturally activate antioxidant and pro-oxidant proteins that would otherwise be quiescent and substrate-limited. This makes the interpretation of oxidative changes in cell culture seductively clear. The measurement of a myriad of reactive oxygen species such as superoxide and hydrogen peroxide and biomarkers of oxidation such as lipid peroxidation is technically simple in cultured cells. However, the judgment that these changes are deleterious in the context of the whole organism is biologically unsound.

### NADPH: The Electron Carrier for Biomass Synthesis, Not for Cell Work

Cycling cells have higher NADPH/NADP<sup>+</sup> ratios than postmitotic cells (Attene-Ramos et al., 2005). Cultured cells must double their biomass each day in preparation for division. Postmitotic cells in tissues do not. Postmitotic cells direct electrons to NADH for cellular work, not NADPH for biomass production. This essential difference between growing and nongrowing cells must be grasped before the different roles of mitochondria in growing and nongrowing cells can be understood. The synthesis of lipids, proteins, DNA, and RNA requires the use of electrons carried by NADPH to make new carbon-carbon and other chemical bonds. NADPH is made in large amounts by the pentose phosphate pathway in which glucose 6-phosphate is used before entering glycolysis to make ribose for DNA and RNA synthesis and NADPH for macromolecular synthesis and glutathione metabolism (Wamelink et al., 2008). When incoming electrons from glucose and other nutrients are directed to NADPH, those electrons are not available for NADH used in mitochondrial oxidative phosphorylation. The combined effect of increased NADPH and hyperoxia (21% O<sub>2</sub>) in cell culture conspires to amplify superoxide and hydrogen peroxide production by NADPH oxidases, making the study of more subtle factors such as regulation of the pentose phosphate pathway by nitric oxide (Bolaños et al., 2008) and compartmental redox regulation during differentiation challenging or impossible.

### Multicellularity and Metabolic Complementarity: The Autonomy of Cells in Culture

In cell culture, investigators necessarily remove the normal connectedness of cells in tissues. As a consequence, single cells in culture must be cell-autonomous, that is, they must synthesize everything they need for growth without

reliance on supplies from other cells. This is not the case in somatic tissues. In tissues, distant and neighboring cells adopt complementary metabolic functions. It is wasteful for photoreceptor cells in the eye, for example, to express genes that are used to produce muscle contraction in the heart. Likewise, it is wasteful and potentially toxic for all somatic cells to make a particular hormone such as insulin or testosterone. In another example, the cells of the liver lobule clearly differentiate along the gradient of oxygen established between the high oxygen present in the vicinity of the hepatic artery in the portal triads where ornithine transcarbamoylase is expressed and the low oxygen present in center of the lobule surrounding the portal vein where ornithine aminotransferase is expressed (Naviaux and McGowan, 2000). Somatic cells epigenetically silence unused genes by DNA methylation and other processes. The process of DNA methylation is regulated by folate, B12, and S-adenosylmethionine metabolism, which are also controlled by mitochondria (Naviaux, 2008). The natural metabolic cooperativity among differentiated cells in the body is lost in cell culture. No longer is there any selective pressure for cells to cooperate as they do in tissues and organs. No longer can the death of a few cells be clearly identified in the context of its evolutionary function to decrease the probability of death of the organism under stress. It is easy to see how an investigator studying cells in a dish might think that active cell processes that cause cell death, such as apoptosis and necrosis, are “bad.” However, it is the pathway’s oxidative shielding, and occasionally cell death, that permits the organism to live on and reproduce under the ever-changing conditions of life on Earth.

*Tug at a single thread in Nature and you will find  
it connected to the rest of the Universe.*

—modified from John Muir (1911) (Muir 1911)

### Cell Metabolism Is Like an Ecosystem

The chemical reactions of the cell take place in a myriad of discrete locations and compartments within the cell that are maintained in thermodynamic disequilibrium. Natural redox boundaries and oxygen gradients are maintained by proteins and membranes in each of these compartments. Cell metabolism can be visualized intuitively as a coral reef ecosystem. The metabolic products of one compartment in the cell are used as resources by other compartments, just as one species of coral can provide resources for another species in the reef. Metabolism is a complex trophic web that stabilizes or destabilizes the differentiated function of the cell. Ultimately, the end products of metabolism are released from the cell into the blood and excreted in the urine, back into the external ecosystem. In both the coral reef and the microcosm of the cell, small-molecule metabolites and signaling molecules drive changes in gene expression, not the reverse. The success of transgenic and gene knockout experiments over the past 20 years has given scientists the impression that genes drive the evolution of metabolism. This is wrong. Rather, it is environmental nutrients and the small molecules of metabolism that drive the evolution of genes.

Over evolutionary time, genes and gene expression patterns evolved to handle the resources provided by the environment. Over shorter time periods of minutes to hours, and

weeks to months, nutrients such as glucose, fats, and amino acids, and small molecules of metabolism are the forcing variables that induce the changes in enzyme activity and gene expression associated with feeding, fasting, and seasonal variations in nutrient availability. The amino acid leucine plays a central role in stimulating the master fuel regulator mammalian target of rapamycin (mTOR) and inhibiting AMP kinase and autophagy (Han et al., 2012).

Many intermediary metabolites act differently inside and outside the cell. Inside the cell they act as carbon skeletons for fat, protein, carbohydrate, DNA, and RNA synthesis. Outside the cell they act as signaling molecules that bind cell receptors and alter gene expression. For example, ATP is an energy-carrying molecule inside the cell. Outside the cell, ATP is a “mitokine” and damage-associated molecular pattern (Zhang et al., 2010) that binds ionotropic and metabotropic purinergic receptors, activating innate immunity and inflammatory pathways (Marques-da-Silva et al., 2011). Succinate is a Krebs cycle intermediate inside the cell, but binds the G protein-coupled receptor 91 (GPCR91) on the cell surface that can reverse the antiplatelet activity of aspirin (Spath et al., 2012). In another example, citric acid inside the cell is the namesake of the “citric acid cycle,” known more commonly as the Krebs cycle. Outside the cell, citrate is a mobile carbon source and barometer of nutrient availability. Citrate is taken into cells via a cell surface transporter called INDY (for “I’m Not Dead Yet”) that when mutated leads to cellular citrate depletion and mimics the life-extending effects of caloric restriction (Birkenfeld et al., 2011). These genetic manipulations illustrate the role of small-molecule metabolites as being prime regulators of cell gene expression. The literature on this topic of metabolic regulation of gene expression is extensive (for a recent review see Buchakjian and Kornbluth, 2010).

### Metabolic Consequences of Nutrient Excess

In 1929, Herbert Crabtree used mouse cancer cells to show that when glucose was added to a medium oxygen consumption decreased (Crabtree, 1929). Mitochondria were not yet identified as the oxygen-consuming particles. Otto Warburg coined the term the “Atmungsferment,” the iron and cytochrome-containing respiratory catalyst, to describe the site of oxygen consumption in cells (Warburg and Negelein, 1928) later discovered to be mitochondria. The Crabtree effect has been called the inverted Pasteur effect, because in the Pasteur effect exposure to oxygen was found to inhibit anaerobic glycolysis. The magnitude of the respiratory inhibition by glucose caused by the Crabtree effect varies between 5 and 50% (Ibsen, 1961) depending on the cell type and the concentration of glucose added. The Crabtree effect plays an important role in many conditions, including diabetes, in which persistently high levels of calories and glucose produce a relative decrease in mitochondrial oxygen consumption. There are several biochemical mechanisms that combine to produce the Crabtree effect under conditions of nutrient loading (Sussman et al., 1980). The most significant is the inhibitory effect of the cytosolically produced [ATP]/[ADP][Pi] ratio on mitochondrial ATP synthesis. This happens because mitochondrial oxidative phosphorylation requires cytosolic ADP and Pi to make ATP. When cytosolic ATP rises and ADP falls, ADP becomes limiting in mitochondria, and the ex-

cesses of cytosolic ATP inhibits the forward action of mitochondrial ATP synthase (complex V) by classic mechanisms of product inhibition. This induces a chemiosmotic backpressure of protons in the mitochondrial inner membrane space and hyperpolarizes the mitochondrial membrane, i.e., makes the mitochondrial membrane potential ( $\Delta\psi_m$ ) more negative. Excess electrons that enter mitochondria under these conditions cannot be used to make ATP because of the backpressure. The partial reduction of oxygen to superoxide and peroxide serves as a “pressure release valve” (Fisher-Wellman and Neuffer, 2012) that permits excess electrons to be dissipated and excess oxygen to be exported from the cell in the form of soluble hydrogen peroxide. All of these biophysical and thermodynamic consequences of nutrient loading result in a net decrease in mitochondrial oxygen consumption that we call the Crabtree effect.

Because mitochondria create the oxygen sink for the cell, when mitochondrial extraction of oxygen is decreased, cell and tissue oxygen levels rise, and the tissue extraction of oxygen from the blood falls. This is observed clinically as a decrease in the arteriovenous difference in  $pO_2$ . Physiologically, this is interpreted as “wasted” oxygenation. Ultimately, it results in the pruning of capillary beds and reductions in tissue vascularity, i.e., peripheral vascular disease. Over time, this leads to chronic tissue hypoxia, ischemia, and loss of organ function, to heart and kidney failure, and chronic neurodegenerative disease.

### Tissue Hypoxia and Ocean Hypoxia: Universal Metabolic Response to Nutrient Loading

There is an ocean-scale analog to the cellular Crabtree effect. When excessive amounts of nutrients are concentrated in agricultural fertilizer runoff and urban waste and carried downriver to the ocean, the metabolism of plankton in the sea is changed. Ultimately, this process creates seasonal and persistent “dead zones” of ocean hypoxia (Diaz and Rosenberg, 2008) that cannot support coral, fish, shellfish, or any eukaryotic life larger than a nematode or a few small snails. Repopulating these lost ecosystems can take years of concerted remediation. Excessive “calories” and nutrients injected into the ocean trigger an ecosystem-scale Crabtree effect, in a biphasic process that is similar to type II diabetes. First, there is an inhibition of aerobic metabolism and a rapid increase in algal and cyanobacterial oxygenic photosynthesis to create plankton blooms. This occurs for the same metabolic reasons as in nutrient-loaded human cells; excess nutrients exceed the oxidative capacity of the cells and redirect these excess electrons to NADPH and biomass synthesis. Nonphotosynthetic cells also shift to this more fermentative metabolism to facilitate rapid conversion of the new nutrient resources to biomass. In diabetes, this shift to biosynthesis leads to organ hypertrophy and the accumulation of adipose tissue and intracellular fat. The net result in the ocean is a transient increase in water oxygen because of photosynthesis and decreased oxygen utilization by heterotrophs. This metabolic shift is quantified by biological oceanographers as an increase in bacterial growth efficiency (Azam and Malfatti, 2007). This first stage of hyperoxia is short-lived and rarely measured. This is similar to the situation in diabetic tissues exposed to excessive nutrients. The second stage of the response to nutrient loading is a decrease in water oxygen. In

the ocean this occurs because the excess plankton biomass produced by the blooms dies and becomes fuel for other bacteria that extract oxygen from the water to process the dead biomass. Hypoxia results. In tissues, chronic nutrient excess leads to tissue hypoxia because of capillary pruning that results from decreased mitochondrial oxygen consumption as described above, and from decreased arteriovenous differences in  $pO_2$ . Initially, the tissue hypoxia from excess nutrient load is patchy, resulting in localized areas of ischemia and segments of dysfunction. Later, the patches of organ hypoxia begin to merge and significant organ dysfunction occurs.

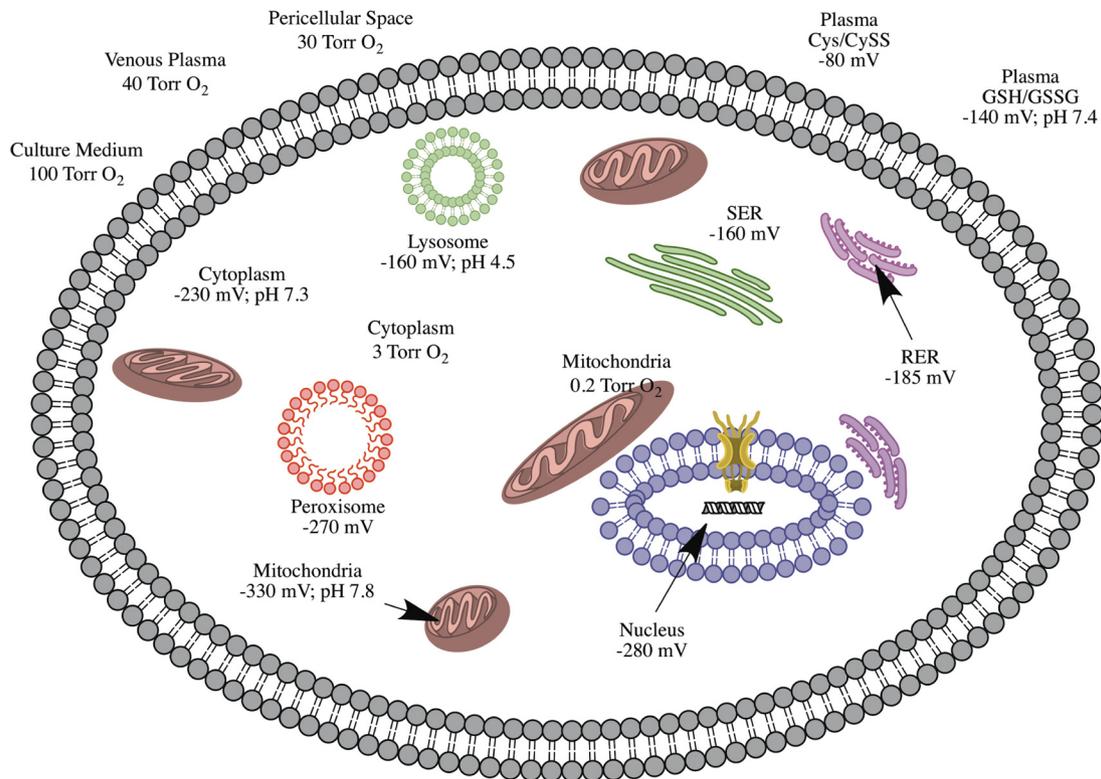
### Redox Compartments and Oxygen Gradients in the Cell

The intracellular “ecosystem” of the eukaryotic cell is comprised of at least eight different organellar compartments, each with its own redox poise (Fig. 1). These include: lysosomes (Gille and Nohl, 2000), smooth ER, rough ER (Hwang et al., 1992), the Golgi (Navas et al., 2010), the cytosol (Go and Jones, 2008), peroxisomes (Yano et al., 2010), the nucleus (Go and Jones, 2011), and the mitochondrial matrix (Go and Jones, 2008). These compartments are not in equilibrium because the proteins that maintain the redox in each compartment, and the membranes that separate them, are not diffusible (Jones, 2010). The standard redox potentials of each of these compartments with regard to the glutathione couple (GSH/GSSG) have been measured (Table 1).

In contrast to sharp boundaries of redox poise that define the different organelles of the cell, the concentration of oxygen at any point in the cell is determined by oxygen diffusion along its gradient from the capillary to the mitochondrial matrix. With a little imagination, one can imagine that each organelle “swims” to a point in the cellular ecosystem that best meets its need for oxygen and nutrients. The oxygen partial pressure of 40 Torr in venous plasma, 30 Torr in the pericellular space (Pittman, 2011), and 0.02 to 0.2 Torr (25–250 nM) in mitochondria (Scandurra and Gnaiger, 2010) can be conceptualized in an “oxygen well” or “target diagram” of the cell (Fig. 2A). When the measured glutathione redox couple is plotted against the log of the partial pressure of oxygen in Torr, a linear regression can be calculated. The formula for this line is: GSH/GSSG redox (in mV) =  $82.6 \times \text{Log}(\text{oxygen in Torr}) - 272$  (Fig. 2B). This connection between oxygen concentration at every point along the oxygen diffusion gradient and compartmental redox helps explain the profound effect that small changes in mitochondrial oxygen consumption, leading to increased dissolved oxygen concentrations in throughout the extramitochondrial compartments of the cell, can have on the metabolic and work performance of the cell. Indeed, the cell cycle has been characterized as a redox cycle (Burhans and Heintz, 2009).

### Cellular Thermodynamics and Work

Ilya Prigogine described the mathematics of dissipative systems in which physical work and the capacity for self-organization can be extracted from the environment by maintaining a compartmentalized, collective chemical “distance” from thermodynamic equilibrium (Prigogine, 1984). Mito-



**Fig. 1.** Redox boundaries and oxygen tensions in the organellar compartments of the cell. Values of oxygen concentration, glutathione redox potential, pH, and associated references are listed in Table 1. Cys/CySS, cysteine/cystine; GSH/GSSG, glutathione/oxidized glutathione homodimer; SER, smooth endoplasmic reticulum; RER, rough endoplasmic reticulum.

TABLE 1

Compartmental redox and oxygen tension in mammalian cells

Glutathione redox potentials vary between 10 and 15% according to growth conditions in single cells and vary up to 20% in different cell types. Plasma redox potentials vary between 5 and 10% in a diurnal cycle and become up to 20 mV more oxidizing with age and disease (Go and Jones, 2011).

Compartment	pH	Glutathione Redox, GSH/GSSG		Dissolved Oxygen
		<i>mV</i>		<i>Torr</i>
Mitochondrial matrix	7.8–8.0	–330 (Go and Jones, 2008)	0.2 (Gnaiger et al., 1995; Scandurra and Gnaiger, 2010)	
Nucleus	7.6	–280 (Go and Jones, 2011)	0.8*	
Peroxisomes	7.6	–270 (Yano et al., 2010)	1*	
Cytoplasm	7.3	–230 (Jones, 2010)	3*	
Rough ER	7.3	–185 (Hwang et al., 1992)	11*	
Smooth ER	7.3	–160 (Jones, 2010)	23*	
Golgi	5.0 (Navas et al., 2010)	–160	23*	
Lysosomes	4.5	–160 (Gille and Nohl, 2000)	23*	
Pericellular space	7.4	–150	30 (Copp et al., 2009)	
Venous plasma	7.3	–140 (Go and Jones, 2008)	40 (West, 2000)	

\* Estimated from the regression equation:  $\text{Log}(pO_2 \text{ in Torr}) = (\text{mV redox} + 272) \div 82.6$ .

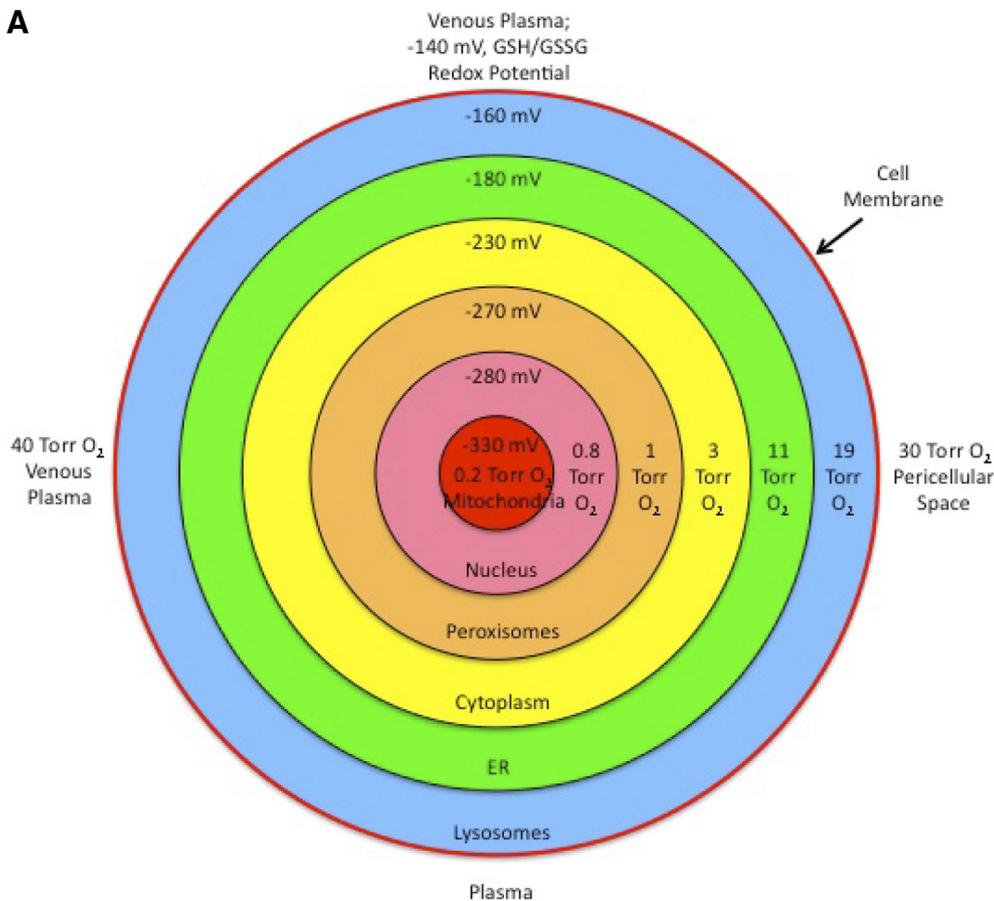
chondria set a limit on the maximum free energy available for work by a cell by consuming oxygen and transforming metabolites. Highly aerobic mitochondria produce a deep oxygen gradient (Fig. 3) of nearly 150-fold, from 30 Torr in the pericellular space to 0.2 Torr (Scandurra and Gnaiger, 2010) at the site of cytochrome *c* oxidase in the inner mitochondrial membrane. The depth of the oxygen sink creates the potential energy that acts like a coiled spring to drive the living clockwork of metabolism, development, differentiation, and organ function.

Less active mitochondria maintain more shallow oxygen gradients and have a lower work capacity, but greater proliferative capacity. This is illustrated in Fig. 3, left. This occurs because electrons in the cytosol are redirected to NADPH and macromolecular synthesis and away from usage as NADH in mitochondria when oxygen consumption rates are lower (Fig. 3).

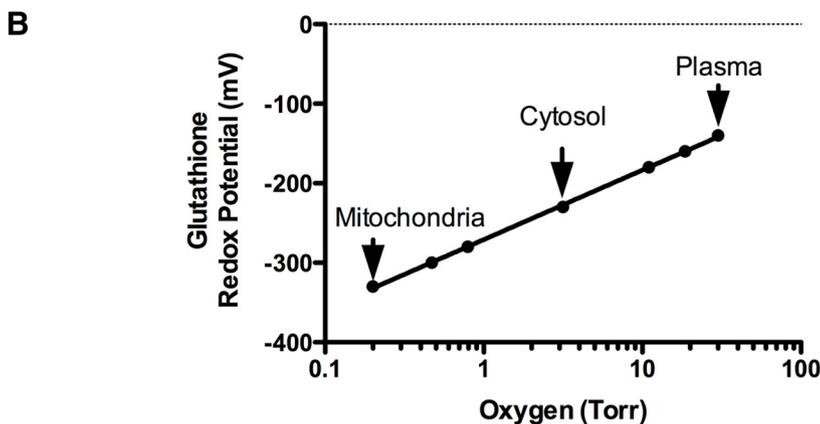
The ultimate energy currency of the cell is electrons that enter the cell in the form of the chemical bonds in the carbon skeletons of nutrients such as glucose, amino acids, and fatty acids. Mitochondrial oxygen consumption determines the depth of the oxygen sink and ultimately tips the redox balance of the cell either toward growth and proliferation or to differentiation and work.

### Metabolic Memory and Exercise

Short-duration physical or chemical stimuli produce long-term cellular effects by inducing kinetically linked chains of events that can be measured metabolically and by protein changes. This phenomenon is called metabolic memory. It is the result of post-translational activation and inhibition and transcriptional activation and inhibi-



**Fig. 2.** Conceptualized oxygen well and redox diagram of the cell. A, oxygen well diagram and redox zones of the cell. This is a schematic that illustrates the measured standard redox potential of the glutathione couple (GSH/GSSG) in six compartments of the cell. B, semilog plot of the partial pressure of oxygen shows a linear correlation with compartmental redox potential. GSH/GSSG redox (in mV) =  $82.6 \times \text{Log}(\text{oxygen in Torr}) - 272$ .



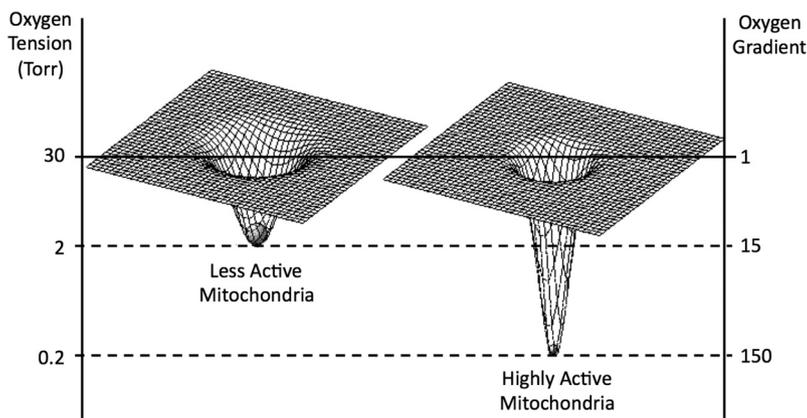
tion events that are initiated by the short-duration stimulus. Many examples are known. The most widely reported example is the long-term effect of short-term, strict metabolic management in patients with diabetes. Even after the management of diabetes is relaxed long-term metabolic changes in insulin sensitivity and decreased incidence of vascular complications and end-organ disease are observed (Cooper, 2009).

Physical exercise is perhaps the best-known method of inducing metabolic memory. Even a single session of exercise will produce an increase in basal metabolic rate for hours after the session (LaForgia et al., 2006). Adaptive strength and cardiovascular benefits can be measured over days. Regular resistance training produces metabolic memory and reverses age-related changes in skeletal mus-

cle over months (LaForgia et al., 2006). The mechanisms of these adaptive changes to exercise seem to require the transient pulses of ROS that are produced (Niess and Simon, 2007).

### Antioxidant Therapy Inhibits the Benefits of Exercise

What happens if the normal amounts of superoxide and hydrogen peroxide produced during exercise are inhibited by treatment with antioxidants? In a ground-breaking study in 2009, Ristow et al. (2009) studied the effect of antioxidant supplements (vitamin C at 1000 mg/day; vitamin E at 400 IU/day) on insulin sensitivity and markers



**Fig. 3.** Mitochondria create the oxygen gradient that generates the thermodynamic potential energy that drives metabolism and cellular work. Right, highly active mitochondria create a deep oxygen sink of 150-fold (30–0.2 Torr) that is highly electrophilic, attracting electrons in the form of NADH like iron to a magnet. This potential energy is harnessed like a coiled spring to power the functional capacity of the cell and provide metabolic reserves that are made available under times of stress. Left, less active mitochondria produce a shallower gradient of 15-fold in this example (30/2 Torr) and have a lower work capacity and less oxidative reserve, but are able to divert electrons more efficiently to NADPH for biosynthesis and cell proliferation.

such as plasma adiponectin and the master mitochondrial biogenesis regulator peroxisome proliferator-activated receptor- $\gamma$  coactivator 1 $\alpha$  (PGC1 $\alpha$ ) after 1 month of exercise. They found that the subjects who exercised and did not take antioxidant supplements had significant improvements in insulin sensitivity, adiponectin, and PGC1 $\alpha$ . Antioxidants inhibited these metabolic benefits of exercise. Ristow et al. proposed that mitochondrial hormesis, or beneficial adaptation to stress, requires transient oxidative stress to induce the downstream changes in nuclear gene expression that promote health and longevity (Ristow and Schmeisser, 2011).

### Antioxidant Treatment Decreases Tissue Perfusion

Experimental work has also been conducted to study the effects of vitamin C and the antioxidant tempol on skeletal muscle performance. When antioxidants were provided acutely, Copp et al. (2009) showed that spinotrapezius muscle contractile force was decreased 25%. Muscle blood flow was also reduced, and the resting oxygen tension in the skeletal muscle was reduced 14%, from 29.9 to 25.6 Torr (Copp et al., 2009). Copp et al. report that optimum blood flow and optimum contractile force depend on the redox state of skeletal muscle. Therefore, antioxidant therapy resulted in decreased muscle perfusion and contractile performance measured acutely (Copp et al., 2009) and interfered with the beneficial effects of exercise such as increased insulin sensitivity when measured after 1 month of supplementation (Ristow and Schmeisser, 2011).

### Phytonutrients and Xenohormesis

Plants and animals that share an ecosystem coevolve. Many plants depend on mammals and birds for seed dispersal. Therefore, the survival of these plants depends on the survival of the mammals and birds that facilitate the dispersal of their seeds. If the coevolving animals do not survive a drought or other seasonal hardship, the plants also perish. Plants have developed secondary metabolites and small-molecule stress hormones such as resveratrol that not only help the plant survive, but also activate molecules such as the sirtuins in animal cells that produce a metabolic shift that improves drought and heat tolerance in the animals. Resveratrol is a polyphenol that activates the lysine-deacetylase activity of sirtuin 1 under conditions of caloric restric-

tion that promote a high NAD<sup>+</sup>/NADH ratio. The principal health benefits of fruits and vegetables lie not just in the vitamins, minerals, and fiber they provide, but in the dozens to hundreds of phytonutrients such as the polyphenols, flavonoids, terpenoids, and quinones that have evolved to activate mammalian cellular targets such as sirtuins, cytoskeleton, tyrosine protein kinases, phosphoinositide 3 kinase, mitogen-activated protein kinases, and transcription factors such as Keap1 and NRF2 (Murakami and Ohnishi, 2012). Animals that consume foliage from stressed plants redirect energy resources away from reproductive pathways. This results in a decrease in fecundity. However, the animals benefit directly from an increase in their own heat and drought tolerance. This phenomenon in which coevolving plants produce signaling molecules that benefit ecologically connected animals is called “xenohormesis” (Howitz and Sinclair, 2008).

An important fact has emerged in the study of the antioxidant properties of plants. Just as in the response to exercise, the durable antioxidant effects of botanicals occur because of their acute, but transient, pro-oxidant (electrophilic and/or ROS-generating) effects (Speciale et al., 2011). Electrophiles abstract electrons from (oxidize) other molecules. Metabolically, this is similar to the action of intracellular microbial pathogens that “steal” electrons to make carbon-carbon bonds and replicate the parasite biomass at the expense of the cell. For this reason, the stereotyped metabolic responses antioxidant defenses and innate immunity are activated similarly by electrophiles and certain microbial pathogens. Potent phytonutrients such as curcumin in turmeric, quercetin in onions, sulforaphane in broccoli, and epigallocatechingallate in green tea produce their antioxidant effects by acting acutely as pro-oxidants in the cell and inducing metabolic hormesis. The electrophilic chemical nature of these compounds up-regulates Keap1/NRF2-mediated transcription of dozens of antioxidant defense genes that contain antioxidant response elements (electrophile response elements). These include enzymes for glutathione synthesis and metabolism ( $\gamma$ -glutamylcysteine synthetase, glutathione peroxidase, glutathione reductase, sulfiredoxin, and thioredoxin), stress proteins such as heme oxygenase-1, and drug detoxification enzymes such as glutathione transferase and NADPH-quinone oxidoreductase, and cytokines such as interleukin-6 (Speciale et al., 2011).

### Conclusions

ROS and oxidative changes in chronic disease are the symptoms of disease and not the cause. Indeed, transient and

regular stimulation of ROS production is required for mitochondrial hormesis and the beneficial physiologic adaptations associated with exercise and a diet rich in fruits and vegetables. Membrane lipid peroxidation, fibrosis, protein oxidation, and hundreds of other markers that were formerly cataloged as oxidative damage need to be understood as cellular oxidative shielding. The conserved pathways of oxidative shielding are protein-catalyzed reactions that evolved as the first steps in innate immunity. They evolved to protect the cell from chemical and microbial threats in the environment. Both oxidative and reductive stress will trigger cellular oxidative shielding. Oxidative shielding takes many forms in different bacterial, plant, and animal phyla. All forms ultimately increase membrane rigidity, decrease membrane permeability, and inhibit cell division. Systemwide conditions such as calorie restriction or nutrient loading modify the cellular response to stress. When stress coincides with dietary nutrient excess, cellular electrons are transferred preferentially to NADPH and not NADH, glycolytically synthesized ATP inhibits mitochondrial oxygen consumption via the Crabtree effect, and chronic inflammation results.

Research efforts need to be redirected. Catalogs of the many forms of oxidative changes that are found in 100 chronic diseases provide no insight into the underlying pathogenesis of disease. Treatment of oxidative changes in chronic disease with antioxidants is similar to treating a fever with aspirin instead of treating the pneumonia. If the clinical outcome selected for monitoring the disease is the fever, then aspirin will be “clinically proven” to be effective at improving the “disease.” However, the untreated pneumonia might still kill the patient. It is better to “treat” the fever by treating the bacterial cause of the pneumonia. Certain antioxidants might be shown to decrease oxidative markers such as nitrotyrosine. However, nitrotyrosine-modified proteins are a symptom, not the disease. More patients will benefit when the true cause of the fever, the cause of the disease, is identified and treated.

New methods of targeted and untargeted metabolomics will help decode the language of metabolism in disease (Tautenhahn et al., 2011; Yanes et al., 2011) and assist physicians in providing personalized medical treatment. NextGen treatments will need to restore the normal “ecology” of metabolism. Complex diseases such as autism, autoimmune disease, diabetes, cancer, and kidney and cardiovascular disease do not have simple causes that are true for every patient. Personalized causes must be sought and new diagnostic tools developed. Identification of the causal chemistry and environmental factors that trigger innate immunity and metabolic memory that initiate and sustain oxidative shielding will lead to fresh new therapies for old diseases.

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