Iron, Free Radicals, and Oxidative Injury

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Iron metabolism and superoxide metabolism are clearly interactive, especially under pathological conditions. Each can exacerbate the toxicity of the other. Iron overload may amplify the damaging effects of superoxide overproduction in a very broad spectrum of inflammatory or ischemia-related conditions. Furthermore, chronic oxidative stress may modulate iron uptake and storage, leading to a self-sustained and ever increasing spiral of cytotoxic and mutagenic events.

IRON IS physiologically essential and biochemically dangerous. The most common cause of infant death by accidental poisoning is the ingestion of ferrous sulfate tablets (1). There has always been a natural tendency to associate iron with strength and well-being, even before biochemists realized that it is a necessary trace element, essential for life itself. The ancient Greeks dissolved iron filings in vinegar, hoping that if they drank this liquor they would acquire the strength of iron. After its recognition as an essential nutrient, the common but faulty assumption followed that "more is better". This assumption persists today, even among some physicians and nutritionists. Accordingly, for several decades we have produced "iron-fortified" foods in an attempt to eliminate iron-deficiency (which afflicts less than 10% of the U.S. population (12)) with little appreciation of what this supplementation might do to the remaining 90% of the populace who are in a state of iron excess. For the majority of persons, iron supplementation simply results in ever increasing stores of excess iron in the body. Evolution has given us mechanisms for absorbing dietary iron with about a 10% efficiency, but, oddly, we have no mechanism for the elimination of excess iron. As a result, cells continuously store excess absorbed iron in a complex with the protein ferritin. This protein is found in all tissues, especially liver. If the body experiences a sudden and significant loss of blood, these stores are drawn upon for the synthesis of new hemoglobin to replace that which has been lost. In modern society, transfusion has supplanted even this need. Hence, excessive iron stores serve no known useful function to an otherwise healthy body.

THE BIOCHEMICAL RISKS OF EXCESSIVE IRON STORAGE

Superoxide is a free radical-- a compound with an odd number of electrons. It is derived from molecular oxygen by the addition of a single electron (16). Reactions that produce superoxide biologically occur under a very broad spectrum of pathological circumstances, including all infectious diseases (3), all inflammatory diseases (41), and all diseases that involve ischemia and reperfusion (40). Phagocytes such as neutrophils and macrophages possess an NADPH oxidase that produces superoxide radical when the cell is activated as a component of the bactericidal armamentarium (3). Many tissues contain the enzyme xanthine dehydrogenase, which may be converted to the superoxide-producing xanthine oxidase following ischemia and reperfusion (40). In addition, it appears that ischemically injured mitochondria become a major source of superoxide radical during post-ischemic reoxygenation (44). Superoxide radical is not the highly reactive species some expect it to be, but its reactions show considerable versatility. It can serve as a mild oxidant, a fairly strong reductant, or as an initiator or terminator of free radical chain reactions. Many key enzymes have been found to be inactivated directly by superoxide (42). These include catalase, creatine phosphokinase, glyceraldehyde-3-phosphate dehydrogenase , glutathione peroxidase, myofibrillar ATPase, adenylate cyclase, and Ca^{2+}-Mg^{2+}-ATPase. Even so, many believe that the most generally destructive action of superoxide radical may be bringing about the reductive release of iron from ferritin (5,7). It has been proposed (6) that $O_2^{\cdot-}$ enters the ferritin-core through the hydrophilic channels, followed by reduction of Fe(III) to Fe(II). This enables the release of iron from the ferritin-core. Iron is a redox-active transition metal, meaning that it may readily oscillate between ferrous, or Fe(II), and ferric, or Fe(III) states, accepting or donating an electron to a variety of biological substances, thereby catalyzing a variety of damaging reactions within the cell. Because of this redox activity, iron is normally handled very carefully by cells and organisms. In the healthy

state, there is never an appreciable concentration of "free" iron (or iron chelated by low molecular weight compounds). Any released Fe(II) is immediately chelated by compounds such as citrate or ADP, but these complexes readily participate in redox reactions, catalyzing the formation of HO˙ or the initiation of lipid peroxidation as discussed below. Most small chelators of iron can accommodate the coordination geometry of either Fe(II) or Fe(III), such that they provide little hindrance to its cyclical reduction or oxidation. The macromolecular chelators of iron such as transferrin and ferritin, on the other hand, provide binding sites of such rigid specificity that Fe(III) is bound extremely tightly, but Fe(II) is not bound at all. During transit from one tissue to another, iron is carried by transferrin. When this iron complex enters a cell via the transferrin receptor, the iron is transferred to and stored in the protein ferritin if it is not immediately needed for another purpose. A very important characteristic of both of these proteins is that they hold their iron in the Fe(III) state. Due to kinetic restrictions as well as the thermodynamics of binding, the iron is very difficult to reduce in transferrin and in ferritin by the usual cellular reductants, and is thus shielded from release and from unwanted redox participation (62).

Why is stored iron potentially dangerous? As long as we remain healthy, our stored iron appears to pose no great problem. Under disease conditions, however, it becomes a significant liability as a result of the multiple possibilities for superoxide production. As shown in Figure 1, it is the storage form of iron, the protein ferritin, that is vulnerable to attack by superoxide radical ($O_2^{\cdot-}$) leading to the release of its stored metal (5-7,23). It may be mentioned at this point that certain other iron-containing proteins within the cell share this vulnerability to superoxide. These proteins, however, contain iron in functional or catalytic settings and serve as enzymes or regulatory proteins rather than storage proteins. Quantitatively, they probably represent less iron than is typically present in ferritin. Aconitase, which exists in cytosolic and mitochondrial forms, contains a 4Fe/4S cluster found in a number of iron-containing proteins. These iron-sulfur centers are readily destroyed by reaction with the superoxide radical with release of iron (17). In vivo, the process is readily reversible with recovery of the iron-sulfur cluster and of the associated activity.

Catalysis of Hydroxyl Radical Production

Once iron has been liberated in the presence of superoxide and its dismutation product, hydrogen peroxide, the hydroxyl radical (HO˙) may be formed by Haber-Weiss chemistry (Figure 1):

$$Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + OH^- + HO^{\cdot}$$

$$O_2^{\cdot-} + Fe^{+3} \rightarrow O_2 + Fe^{+2}$$

$$\overline{O_2^{\cdot-} + H_2O_2 \rightarrow O_2 + OH^- + HO^{\cdot}}$$
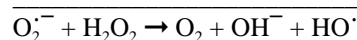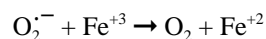
Unlike superoxide radical, which is not highly reactive compared to most other free radicals, the hydroxyl radical is an extremely powerful oxidizing species. In oxidizing potential it is second only to atomic oxygen. It may be produced by the radiolysis of water, and is thus responsible for most of the damage resulting from exposure to ionizing radiation. It came as a surprise to many biochemists that hydroxyl radical could be produced by biological systems themselves, by the simple generation of superoxide in the presence of redox-active iron and hydrogen peroxide (43). This hydroxyl radical can attack all classes of biological macromolecules. It can depolymerize polysaccharides (39), cause DNA strand breaks (22), inactivate enzymes (69), and initiate lipid peroxidation (19). Because lipid peroxidation is a chain reaction that is geometrically amplified by redox active iron, it is this action of hydroxyl radical that may have the greatest pathophysiological consequences in diseases such as ischemic heart disease and stroke.

The Initiation of Lipid Peroxidation

The hydroxyl radical is capable of abstracting an allylic hydrogen atom (H˙) from a polyunsaturated fatty acid chain of a phospholipid molecule (LH):

$$HO^{\cdot} + LH \rightarrow H_2O + L^{\cdot}$$
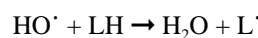
The carbon-centered lipid radical that results (L˙) immediately reacts with molecular oxygen to create a lipid dioxyl radical (LOO˙):

$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot}$$
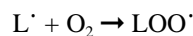
This lipid dioxyl radical is capable of abstracting a hydrogen atom from another polyunsaturated fatty acid chain, creating another L˙ radical which propagates the chain reaction:

$$LOO^{\cdot} + LH \rightarrow LOOH + L^{\cdot}$$
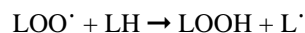
In vivo, it is estimated that a single initiating event (such as the generation of a single HO˙ radical) creates a chain of 10 to 15 cycles before the chain is terminated, usually by reaction with a vitamin E molecule. The effect of one such chain reaction would be the accumulation of 10 to 15 lipid hydroperoxide (LOOH) molecules. This local area of the cell membrane is now "seeded" with the
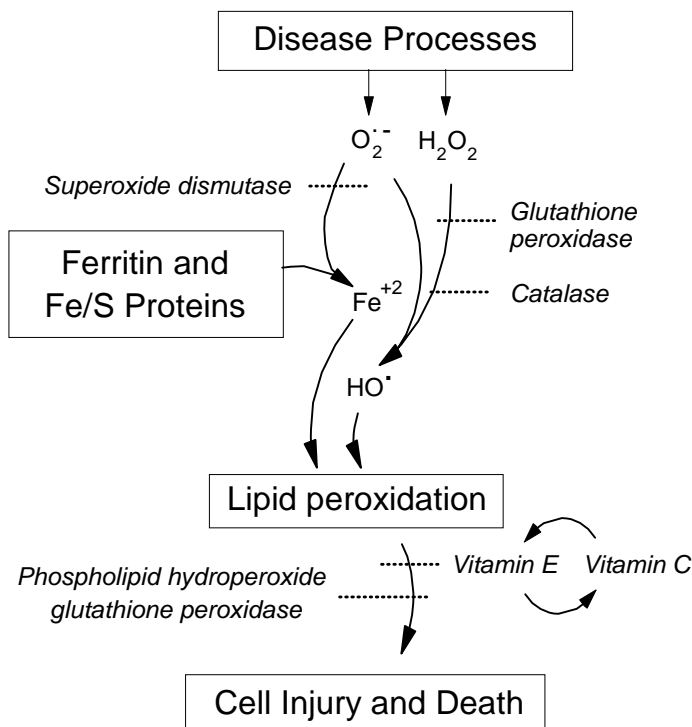
Fig 1. How the production of $O_2^{\cdot-}$ and H_2O_2 leads to the release of iron, to lipid peroxidation, and to cell injury and death. The protective antioxidant enzymes and vitamins are shown in italic, and their points of intervention in the process are indicated by dotted lines.

makings of 10 to 15 new chain reactions because redox-active iron, reduced to the ferrous state by reaction with superoxide, is capable of reducing the hydroperoxide to form a new radical, the alkoxy radical ($LO^{\cdot}$), which is capable of initiating a new chain reaction as above:

$$Fe^{2+} + LOOH \rightarrow Fe^{3+} + LO^{\cdot} + OH^{-}$$

$$LO^{\cdot} + LH \rightarrow LOH + L^{\cdot}$$
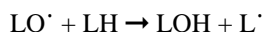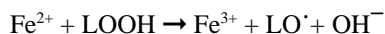
Hence, the presence together of superoxide radical and redox-active iron can be devastating to the cell in terms of maintaining membrane structure and function, and therefore viability. Figure 1 also shows the cellular defense mechanisms that act to prevent this sequence of events. The antioxidant enzymes superoxide dismutase, catalase, and glutathione peroxidase act as a first line of defense to intercept the active oxygen species directly. If these defenses are overwhelmed and events progress to lipid peroxidation, a second line of defense exists. The antioxidant enzyme phospholipid hydroperoxide glutathione peroxidase acts to eliminate peroxidized membrane components by reducing peroxides to alcohols. This action prevents the initiation of new chain reactions by ferrous iron as described above. The

antioxidant vitamins E and C also collaborate to terminate chain reactions, stopping further accumulation of peroxides. When all these defenses are either overwhelmed or consumed, cell membranes may be so damaged that the cell dies.

Species Variability

Recent evidence suggests that this superoxide-dependent iron release from ferritin is species specific (23). The efficiency of iron release was highest for human liver ferritin, being about 3-fold greater than for rat liver ferritin. Horse spleen ferritin showed no superoxide-induced iron release at all. In addition, the methods of purification (commercial source) and storage of ferritin may dramatically affect its behavior with regard to iron release (51). The recognition of these facts may clear some of the confusion in the literature concerning the ability of $O_2^{\cdot-}$ to release iron from ferritin. Reports finding little or no superoxide-dependent release of iron from ferritin have most often studied horse ferritin. Clearly, all future laboratory experimentation in this area should take species variation into consideration. Extrapolation to humans from animal models or test tube experiments should be undertaken with care. Such

species variation has not been reported with regard to the release of iron from proteins that contain iron-sulfur clusters.

Iron Stores May Be Delocalized by Other Pathological Insults

Certain pathogenic agents other than superoxide can also effect the liberation of iron from ferritin or from proteins containing iron-sulfur clusters. Exposure of ferritin to ionizing radiation (52), to redox-cycling xenobiotics such as paraquat, adriamycin, and alloxan (51), or to high levels of nitric oxide (53) can liberate the iron. Iron-sulfur clusters are sensitive to superoxide or peroxynitrite, but not to nitric oxide (26).

THE PATHOPHYSIOLOGICAL SEQUELAE OF IRON-MEDIATED OXIDATIVE DAMAGE

Excess iron may be quite toxic, even to a healthy organism. In the presence of oxidative stress associated with inflammation or ischemic injury, the toxicity is amplified by the mechanisms described above. The ability of iron to worsen oxidative injury can be easily demonstrated in the laboratory. Hearts from iron-loaded animals suffer substantially more injury when subjected to ischemia/reperfusion (in simulation of a heart attack) than do hearts from normal animals (66). Reperfusion injury manifests in many ways, including release of marker enzymes such as creatine phosphokinase (4), appearance of conjugated diene products of lipid peroxidation (36), or loss of contractility (66). It may be shown that ischemia/reperfusion causes iron to be delocalized or redistributed (27). Many studies have implicated an active role for liberated iron in reperfusion injury by demonstrating the protective effect of desferrioxamine (deferoxamine) (8,15,48). This powerful iron chelator has an enormous affinity for Fe(III) over Fe(II), thereby preventing the reduction of the iron it holds, preventing its participation in redox chemistry.

OXIDATIVE STRESS, MODULATATION OF IRON METABOLISM, AND CARCINOGENESIS

Iron greatly exacerbates the injury caused by oxidative stress by the mechanisms outlined above (54). Iron and superoxide have been shown to initiate lipid peroxidation (19) as well as DNA damage (63). Because of the interaction between iron and free radicals it is not surprising that interactive mechanisms of metabolic regulation have evolved between the two. Touati et al. (63) have elegantly demonstrated this interaction in *Escherichia coli*. The bacterium produces a ferric uptake regulatory (Fur) protein that can reversibly bind iron, and serves as an indicator of iron status and availability within the organism. When the Fur protein detects adequate iron (i.e., its iron-binding site is filled), it represses both iron assimilation and the expression of manganese superoxide dismutase (SOD). Conversely, when its site is empty, it upregulates both iron uptake and SOD expression. By inference, the active uptake of iron sensitizes the cell to superoxide. Mutations in the *fur* gene lead to constitutive uptake of iron resulting in iron overload, oxidative stress, and DNA damage including lethal mutations. Overexpression of SOD in these mutants provides partial protection. In SOD-deficient mutants, aerobic growth becomes lethal, but this lethality may be suppressed by mutations that interfere with iron transport, implying that superoxide-mediated iron reduction is responsible for the toxicity.

Iron uptake and disposition by mammalian cells is controlled by a protein called *iron-responsive element binding protein*, or IRE-BP (33). IRE-BP contains an iron-sulfur center, which, when intact, renders the protein inactive. When iron becomes scarce the IRE-BP loses its iron and becomes an active RNA-binding protein. It binds to ferritin mRNA, impeding translation and thus decreasing the cell's production of this iron storage protein. It also binds to the mRNA for transferrin receptor, stabilizing the transcript and thereby increasing production of transferrin receptor. The increased number of receptor molecules increases the cell's uptake of iron.

The iron-sulfur cluster found in IRE-BP is sensitive not only to iron availability, but also to exposure of cells to oxidants such as hydrogen peroxide (37). There are no data on the interaction of superoxide *per se* with IRE-BP, but superoxide is known to inactivate aconitases (26), and the cytosolic aconitase is actually identical to IRE-BP (32). If the iron-sulfur cluster is intact the protein has aconitase activity; if the iron-sulfur cluster is disrupted, it becomes an RNA-binding protein (IRE-BP) (20). One may speculate that cellular overproduction of $O_2^{\cdot-}$ or underproduction of SOD might result in the activation of IRE-BP, resulting in turn in increased uptake *but decreased storage* of iron.

We and others have shown that exposure of cultured cells to low concentrations of the redox-cycling, superoxide-producing herbicide paraquat creates a condition of oxidative stress (47,67). This compound is reduced by the mitochondrial electron transport chain. It then transfers its electrons to molecular oxygen to generate superoxide radical. Cells exposed to paraquat have decreased total content of NADH and NADPH and increased content of malondialdehyde, an end-product of lipid peroxidation (58). They show increased heat sensitivity (60). They also show a higher percentage of apoptotic cells, with increased evidence of DNA fragmentation (McCord and Nelson, unpublished data).

Furthermore, paraquat is known to be an "oxidative mutagen" that increases the frequency of intrachromosomal recombination in a dose-dependent manner (9), even though its product, the superoxide radical, does not react directly with DNA (21). Rather, it is thought that the DNA damage requires the presence of both $O_2^{\cdot-}$ and redox-active iron. These findings imply that the cancer-causing potential of paraquat (29) or of neutrophil-generated superoxide (30) is not due solely to direct effects of superoxide. Carcinogenesis may be promoted most efficiently when two conditions obtain: 1) increased production (or decreased scavenging) of $O_2^{\cdot-}$, and 2) increased cellular uptake, decreased storage, and increased availability of redox-active iron brought about secondarily by increased superoxide.

Both iron (57,64) and oxidative stress (65) have clearly established relationships to mutagenesis and carcinogenesis. Chronic inflammation involves continuous influx of large numbers of activated neutrophils and macrophages that are producing superoxide. Chronic inflammation often leads to carcinoma, with a notable example being the very high risk of colon carcinoma in patients with chronic inflammatory bowel disease (2).

IRON, FREE RADICALS, AND DISEASES ASSOCIATED WITH AGING

A peculiar aspect of iron metabolism is the fact that we have a specific mechanism for its absorption (regulatable to a degree) but we have no mechanism to eliminate excess iron. In fact, loss of blood is the only way our bodies lose iron. As a result, the iron stores of American males increase almost linearly with age (12). Females, due to menstruation, are in relatively good iron balance until the age of menopause, after which time they begin to accumulate iron at a rate comparable to that of males. Sullivan (59) has suggested that it is this difference in levels of stored, excess iron that accounts for the gender difference observed in the mortality statistics for ischemic heart disease.

Human clinical data support the concept that high iron stores impose additional risk from many types of disease. An ongoing study by Salonen et al. (55) identifies high levels of stored iron as a clear risk factor for heart disease. In a study of patients with small cell carcinoma of the lung, Milman et al. (45) found that those patients with the lowest serum ferritin levels at the time of diagnosis had significantly longer survival times. Because, as already mentioned, oxidative stress is associated with nearly all forms of disease, one might expect that iron stores would become a liability to almost all critically ill patients. Because iron stores increase with age, one might then expect that the ravages of disease will be amplified in the elderly, relative to the young.

Iron, Free Radicals, and Neurodegenerative Diseases

A special class of age-related diseases is the neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis.

Parkinson's disease is a degenerative disease of the nervous system characterized by involuntary tremulous motion and decreased muscular power. The senses and intellect are not affected. Onset is in middle or late life, with gradual progression and prolonged course. Familial incidence is only 1 or 2 percent. Considerable evidence implicates iron in the pathogenesis of Parkinson's disease (18,24,46,68). Increased levels of iron were found in the substantia nigra pars compacta when compared to those of normal control patients (68), but the mechanism is unknown. Population based study of Parkinson's disease shows that it affects men more frequently than women (38), consistent with possibility that iron accumulation is a predisposing factor. The nigra may be particularly vulnerable to iron due to its high rate of dopamine oxidation via monoamine oxidase to form hydrogen peroxide (10,14). Oxidative stress has been associated with nigral cell death (31) and increased levels of lipid peroxidation have been found in substantia nigra in patients with Parkinson's disease (13).

Prior to 1983 there was no animal model of Parkinson's disease, and no mechanistic hypothesis as to its etiology. A bizarre occurrence in the California drug culture provided both when several young heroin abusers developed the clinical symptoms of severe Parkinson's disease (35). All had injected a synthetic product, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a by-product of the synthesis of 1-methyl-4-propionoxypyridine, a "designer drug" with heroin-like effects. The compound was found to be oxidized by monoamine oxidases to 1-methyl-4-phenylpyridinium (MPP^+), a potent inhibitor of the NADH dehydrogenase of mitochondrial complex I (56). Hasegawa et al. (25) found that the MPP^+-poisoned complex I became a site for superoxide production. MPTP can also catalytically generate superoxide radical in an iron-dependent reaction (49) and can short-circuit mitochondrial electron flow in a manner similar to paraquat (34), thereby producing increased amounts of superoxide and lipid peroxidation. Strong evidence of free radical involvement in the neurotoxic effects of MPTP came from the demonstration that transgenic mice overproducing SOD are dramatically resistant to MPTP toxicity (50). Other animal studies have been conducted with 6-hydroxy dopamine (6-OHDA), which selectively destroys the nigrostriatal

dopamine pathway in the substantia nigra (28). 6-OHDA reacts with molecular oxygen to produce superoxide anion radical, hydrogen peroxide and hydroxyl radicals (11). MPTP-treated African green monkeys, which showed obvious contralateral hemiparkinsonism, had significantly elevated iron (Fe^{+2} and Fe^{+3}) in the substantia nigra compacta (61). Three times more iron was found not only in the degenerating dopamine cells themselves but also in the surrounding matrix and glial cells. Similar results were reported by Mochizuki et al. (46) who have also shown a good correlation of iron accumulation with loss of dopaminergic neurons. These results suggest that the aberrant metabolism of iron in Parkinson's disease may be secondary to increased oxidative stress, but emphasize once again that iron metabolism and free radical metabolism are interactive.

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