Serial Review: Iron and Cellular Redox Status
Guest Editor: Mario Comporti

MOLECULAR BASES OF CELLULAR IRON TOXICITY

JOHN W. EATON and MINGWEI QIAN
Department of Medicine and James Graham Brown Cancer Center, University of Louisville, Louisville, KY, USA

(Received 9 October 2001; Accepted 1 February 2002)

Abstract—Patients with hereditary or secondary hemochromatosis are liable to cardiac and hepatic failure, and type II diabetes. Despite the highly likely conjecture that iron-mediated tissue damage involves the conspiracy of cellular oxidizing and reducing equivalents, the pathophysiologic events have not been fully elucidated. These latter likely involve toxic effects of iron on intracellular organelles, in particular, mitochondria and lysosomes. The tissues at risk—heart, liver, and pancreatic beta cells—all have highly active mitochondria, which incidentally generate activated oxygen species capable of causing synergistic toxicity with intracellular iron. This suggests the general concept that iron may be preferentially toxic to cells with high mitochondrial activity. At least part of the long-term toxicity may involve iron-mediated oxidative damage to the mitochondrial genome with an accumulation of mutational events leading to progressive mitochondrial dysfunction. An alternative—and not mutually exclusive—mechanism for cellular iron toxicity involves iron-catalyzed oxidative destabilization of lysosomes, leading to leak of digestive enzymes into the cell cytoplasm and eventuating in apoptotic or necrotic cell death. © 2002 Elsevier Science Inc.

Keywords—Hemochromatosis, Mitochondria, Iron, Free radicals

CHRONIC IRON OVERLOAD: DAMAGE TO CELLS AND TISSUES

Organ damage arising from chronic iron overload is remarkable for the range of tissues affected (both replicative and nonreplicative) and for the often slow and insidious onset of organ dysfunction. Foremost amongst the organs and cell types affected by iron overload are the liver, heart, and pancreatic beta cells. Hepatic disease (e.g., cirrhosis and hepatoma) accounts for a large number of the premature deaths amongst patients with hereditary hemochromatosis [1], whereas heart failure predominates in patients with secondary (usually transfusion-induced) hemochromatosis [2,3]. Both groups share a substantially increased risk for development of diabetes in comparison with the normal population [3].

In the case of the heart, the early accumulation of iron is seen predominantly in the epicardium [4]. As overload progresses, the stainable iron tends to have a sarcoplasmic localization but, to the best of our knowledge, the precise subcellular distribution of iron has not been well defined. Diastolic dysfunction appears early in the course of iron overload while systolic dysfunction occurs very late [5]. The possibility that redox reactions driven by highly active mitochondria are involved is supported by observations that other transition metals, such as copper, may engender similar cardiac damage. In Wilson’s disease, which is characterized by delocalization of copper, there is copper deposition in mitochondria [6] and, although the majority of patients do not have co-existent cardiac disease, > 30% of patients with Wilson’s disease do have cardiac problems such as left ventricular hypertrophy [7]. It may be pertinent that the cardiac effects of
iron deficiency show a strange similarity to those of iron overload. Although these effects could be secondary to the anemia per se, it is interesting that, in iron-deficient rats, there is a marked cardiac hypertrophy (especially, left ventricular) along with enlarged mitochondria with abnormal matrices and deficient succinate dehydrogenase activity [8]. This paradox might be resolved if, as suggested below, the pathologies that occur at both extremes of abnormal iron balance reflect mitochondrial dysfunction arising either from iron deficiency (which directly interferes with mitochondrial function) or iron excess (which, as argued below, might arise from mitochondrial dysfunction secondary to accumulated damage to mitochondrial DNA).

In contrast to the cardiac effects of iron overload, there is less information concerning the effects on hepatic tissue and pancreatic beta cells. Iron-induced cirrhosis is characterized by fibrotic changes that precede full-blown hepatic failure. The precise cause(s) of this fibrosis is, however, unknown. Iron may be especially toxic to pancreatic beta cells for several reasons. These cells have intrinsically poor antioxidant defenses and may take up more iron than most other somatic cell types [9]. Probably reflecting these characteristics, beta cells are exquisitely sensitive to diabetogenic redox drugs (such as alloxan and streptozotocin) and—even when intracellular iron levels are normal—the toxicity of these drugs can be blocked by iron chelators such as desferrioxamine [9].

**HOMEOSTATIC RESPONSES TO OXIDATION/IRON OVERLOAD**

Early in the course of iron overload, numerous homeostatic mechanisms likely prevent damage from the accumulating iron. These include the induction of ferritin, which can, to an extent, limit the availability of redox active iron and suppress iron-catalyzed oxidant damage to cells [10,11]. Unfortunately, cells cannot perpetually accumulate ferritin and ultimately this homeostatic mechanism may fail. Similarly, some antioxidant enzymes may be induced when cells are exposed to the oxidant challenge of “free” iron coupled with endogenous redox agents. However, as a general rule, increments in individual antioxidants and/or antioxidant enzymes may afford little protection against oxidant damage that is promoted by transition metals and may even enhance such damage (reviewed in [12]). For example, cellular reducing species such as reduced glutathione may actually promote iron-mediated oxidative reactions (e.g., [13]). In fact, Giulivi and Cadenas [14] have reported that H₂O₂-mediated damage to the DNA of isolated mitochondria is actually decreased by prior GSH depletion and, in isolated nuclear DNA, increased by GSH addition. Thus, although GSH is certainly an antioxidant, it can have pro-oxidant effects when acting as part of a (presumably metal-catalyzed) redox couple.

The exception to the lack of importance of antioxidant enzymes may involve mitochondrial manganese superoxide dismutase (MnSOD). Brown et al. [15] report that rats fed carbonyl iron show significant elevations of hepatic MnSOD activity and this also is seen in a variety of experimental settings wherein mitochondrial oxidation is increased. Furthermore, mice deficient in MnSOD with normal iron balance exhibit progressive mitochondrial damage as reflected by deficiencies in iron-sulfur enzymes (complexes I, II, and III and mitochondrial aconitase), an interesting parallel with the abnormalities found in the iron-loaded mitochondria present in Friedreich ataxia (vide infra) [16–18]. Importantly, in MnSOD-deficient mice there is also an accumulation of oxidative damage to mtDNA [18] and the mice homozygous for MnSOD deficiency die very early and exhibit dilated cardiomyopathy [16,19].

**TARGETS OF IRON-DRIVEN CELLULAR DAMAGE**

Given the tendency of transition metals such as iron to amplify oxidant damage, the fact that organs with very active mitochondria are targeted in iron overload disorders is probably no coincidence. There is little evidence of iron per se exerting toxic effects in the absence of agents that affect the active oxidation and reduction of the metal. Thus, “antioxidants” or chelators which keep iron in one valence (such as Fe³⁺) will effectively block iron-mediated oxidant damage [20]. Similarly, iron-chelating agents that occlude all six coordination positions of the metal also will prevent iron-catalyzed oxidation reactions [21]. Therefore, one reasonable assumption is that iron-mediated cellular damage requires the active conspiracy of “free,” redox-active iron and biological oxidizing and reducing agents.

The liver and heart have high steady-state production of O₂⁻ and H₂O₂ [22], largely derived from mitochondrial activity [23]. The pancreatic beta cell is also rich in mitochondria and is highly sensitive to oxidant-generating xenobiotics (such as alloxan and streptozotocin) and exogenous oxidants [24,25]. Where they have been done, histopathologic and ultrastructural studies of the hearts of patients with iron overload often reveal swollen mitochondria having an electron dense matrix and ruptured mitochondrial membranes (e.g., [26]). Although the hepatic, cardiac, and pancreatic beta cell pathologies caused by iron overload probably involve an iron-driven oxidative component, it is as yet unknown precisely what goes wrong. However, it is notable that ultrastructural abnormalities similar to those observed in iron overload, including mitochondrial swelling and disintegrating cris-
tate, have been observed in the hearts of rats treated with the anthracycline analogue mitoxantrone [27] and in the pancreas of rats fed with dietary carbonyl iron and ethanol [28]. In all cases, metal-catalyzed oxidation reactions have been suggested.

Despite many years of research on the subject, the precise mechanisms through which reactive iron conspires with oxygen to amplify cellular oxidant damage remain unknown. The fundamental of iron-mediated oxidant damage may be the tendency of “free” iron to associate with oxidizable targets within cells (e.g., polyunsaturated fatty acids, proteins, or DNA) and to engage in site-specific oxidation reactions. These latter have often been ascribed to the tread “hydroxyl radical,” but more likely represent tripartite reactions arising from complexes of iron bound to particular target molecules and oxidants which, upon reaction with the iron, form a highly oxidizing intermediate (ferryl or perferryl), which then attacks the substrate.

Polyunsaturated fatty acids

In in vitro models of cell damage and death arising from a combination of iron load and exogenous oxidant (such as H₂O₂), it is likely that peroxidation of polyunsaturated fatty acids (PUFA) within membrane phospholipids is a crucial event. In short-term experiments, antioxidants that prevent PUFA oxidation also will block cell death [29–31]. In experimental models of iron overload in vivo, greatly increased PUFA oxidation of hepatic mitochondria, as well as lysosomal fragility, also have been observed [32]. Interestingly, Bacon and colleagues [33,34] observed that, following oral loading of rats with carbonyl iron, mitochondrial lipid peroxidation occurred at hepatic iron concentrations one half to one third those necessary to trigger microsomal lipid peroxidation. In further experiments, they found that this was accompanied by substantial decrements in mitochondrial metabolism, which could neither be prevented by α-tocopherol supplementation nor worsened by concordant α-tocopherol deficiency [35–37]. Overall, these observations suggest that mitochondrial PUFA are a preferential target for iron-driven peroxidation but that, at least in this model, peroxidation and mitochondrial dysfunction occur independently of α-tocopherol status. In contrast, in an in vitro system involving iron loading of primary cultures of newborn rat cardiomyocytes, similar iron-mediated PUFA oxidation and mitochondrial dysfunction have been observed. In this case, however, supplementation of cells before or after iron loading with α-tocopherol abolished iron-mediated inhibition of both NADH-cytochrome c oxidoreductase (complex I–III) and succinate dehydrogenase [38].

DNA

There is abundant evidence that, in addition to synergizing the oxidation of PUFA, “loose” intracellular iron also will promote DNA damage. Early work by Cochrane and colleagues clearly established that oxidant-induced damage to naked DNA and intracellular DNA is greatly enhanced by iron [39,40]. In the absence of transition metals such as iron and copper, DNA is quite unreactive with oxidants such as H₂O₂. However, in the presence of added iron, DNA scission occurs (e.g., [41]), preferentially in inter-nucleosomal linker regions [42], producing “ladders” resembling those typical of apoptosis [43]. The damage to DNA caused by iron + oxidants is decreased or absolutely prevented by effective iron chelators (i.e., chelators, such as desferrioxamine, which fill all six coordination positions and make the iron chemically unreactive [21]). The known products of reactions between DNA, iron, and oxidants are not yet fully elucidated but include strand breaks, oxidatively modified bases, and DNA-protein cross-links [44,45]. Even lipid peroxidation products have been shown to form covalent products with DNA [46]. Enhanced levels of bulky DNA lesions have been detected using 32P-postlabeling in hepatic DNA from patients with hereditary hemochromatosis [47]. However, the relative importance of all these products in long-term iron-mediated DNA damage is not yet known.

Proteins

Once again, as is true of PUFA and DNA, proteins are resistant to damage by simple oxidants such as H₂O₂ unless transition metals are present. Metal-catalyzed damage to proteins includes loss of histidine residues, bityroine cross links, oxidative scission, the introduction of carbonyl groups (through, e.g., oxidative deamination), and the formation of protein-centered alkyl, alkylperoxyl, and alkoxyl radicals [48]. With a few exceptions, protein damage is likely to be a repairable and nonlethal event for a cell. However, Sohal and colleagues have presented convincing evidence that two mitochondrial proteins—aconitase and adenine nucleotide translocase—may be important targets of long-term oxidative damage [49,50].

MITOCHONDRIA AS TARGETS OF IRON-MEDIATED DAMAGE

One tenable hypothesis concerning the etiology of cell and organ damage arising from iron overload is that excess iron selectively targets mitochondria and, perhaps, the mitochondrial genome. The mitochondrial electron transport chain “leaks” 1–2% of its electrons into
O\textsubscript{2}/H\textsubscript{2}O\textsubscript{2} (probably through the intermediacy of ubisemiquinone at least in the case of complex III [51]). This leak of electrons is responsible for \~90\% of the activated oxygen generated by most cell types. Therefore, to the extent that mitochondria within cells of iron overloaded animals also are exposed to elevated iron, it would appear likely that these organelles would be most extensively damaged.

Experimental iron loading of cultured (beating) rat myocardial cells causes a decrease in membrane PUFA content, loss of thiol-dependent enzyme activities, decreased ATP, and increased lysosomal fragility [52–54]. Similar changes in mitochondrial energy production with iron loading also have been observed in the livers of rats with chronic iron overload [55]. In addition, in the cultured rat myocardial cell model there is a substantial inhibition of NADH-cytochrome c oxidoreductase (complex I–III) and succinate dehydrogenase, both preventable by \alpha\textsubscript{-}tocopherol [38,56]. We should note, however, that, although the sparing effect of \alpha\textsubscript{-}tocopherol does argue in favor of PUFA oxidation as an important factor, it does not mean that the toxicity derives just from membrane damage (as opposed to possible clastogenic actions of oxidizing PUFA; e.g., [46]). It also is worth noting that this iron loading has almost no effect on ubiquinol cytochrome c oxidoreductase (complex III), thought to be an important source of electron leakage [51,57], raising the possibility that mitochondrial iron damage might paradoxically enhance the production of activated oxygen and, thereby, worsen the problem.

**MITOCHONDRIAL DNA: A FRAGILE GENOME**

As discussed above, iron-mediated PUFA oxidation is clearly important in acute, in vitro models. However, the nature of cellular damage secondary to chronic iron overload remains to be established and may or may not resemble that caused by short-term iron loading of cultured cells. From experiments of nature such as Friedreich ataxia, we know that the abnormal accumulation of iron within mitochondria is associated with extensive and irreversible damage. Furthermore, \~10\% of affected patients develop diabetes and \~70\% exhibit a hypertrophic cardiomyopathy [58]. In this congenital disorder, the mutant protein, frataxin, is targeted to the mitochondrial inner membrane and apparently functions to maintain intramitochondrial iron balance [59,60]. Affected mitochondria show not only iron accumulation but also inactivation of iron-sulfur enzymes—complexes I, II, and III and aconitase [61]. Interestingly, similar damage to mitochondrial metabolism results from iron loading of normal myocytes in vitro [56] and hepatocytes in vivo [55]. Furthermore, yeast in which the frataxin homologue is deleted show distinct iron-mediated mitochondrial damage [60].

The possibility that accumulating damage to mtDNA might be involved in the pathologic consequences of iron overload and normal aging is supported, at least indirectly, by a number of observations.

- Damage to mtDNA does, apparently, accumulate in vivo as a function of age. Using full-length PCR, Melov et al. [62] have found a startling absence of full-length PCR products in skeletal muscle from most older humans whom they studied (average age \~70 years) while similar preparations from younger donors (average age \~25) had abundant amounts of the expected full-length product. Kovalenko et al. [63] have made similar observations but also find that the total amount of mtDNA does not decrease as a function of age. These age-dependent changes in the mitochondrial genome have recently been reviewed [64].
- Perhaps equally important are recent observations on age-dependent mtDNA deletions in human cardiac tissue [65]. These indicate that mtDNA damage (at least, deletions) accumulates throughout life and that new deletions are superimposed on prior deletions.
- Compared to nuclear DNA, mtDNA is much more sensitive to oxidant damage [66,67]. In fact, at very low fluxes of exogenously generated hydrogen peroxide, only mtDNA damage accumulates and nuclear DNA is unscathed [67].
- Even in nonoxidant challenged cells, the steady-state level of oxidized bases in mtDNA is 10–15 times higher than in nuclear DNA [68–70] (although we should mention that Hegler et al. [71] argue that many estimates of steady-state mtDNA oxidative modifications have been exaggerated). This accords with the observation that, in the course of evolution, the mutation rate in mammalian mtDNA has been 5–10 times greater than in nuclear DNA [72].

There may be several reasons for the apparent fragility of mtDNA, which include: (i) Mitochondria generate reactive oxygen species. (ii) Mitochondria are intrinsically rich in iron, some of it easily released by oxidants from, e.g., iron-sulfur centers. It has been argued—albeit indirectly—that because DNA is unreactive with H\textsubscript{2}O\textsubscript{2} in the absence of iron (or another transition metal), the preferential damage to mtDNA probably reflects the presence of larger amounts of reactive iron within even the normal mitochondrial [66]. (iii) MiDNA is deficient in histones that may normally provide partial protection against oxidant damage. (iv) Repair of damage to mtDNA is slower and less effective (depending on the types of damage involved) (e.g., [73]).
MIGHT ACCUMULATED DAMAGE TO mtDNA CAUSE ORGAN DYSFUNCTION?

A final important point concerns the question of why mutations in mtDNA might lead to dysfunction of organs such as the heart. In this instance, the pertinent experiments of nature are inherited mitochondrial diseases (such as myoclonic epilepsy and ragged red fibers [MERRF] and mitochondrial myopathy, encephalomyopathy, lactic acidosis, and stroke-like episodes [MELAS]). In these cases, the mitochondrial defects (caused by point mutations interrupting the function of single mitochondrial genes) are heteroplasmic (i.e., present on some but not all copies of mtDNA). In some instances, individuals in whom 50% of mtDNA carries one of these mutations may have no symptoms whatsoever. However, when the frequency of a dysfunctional gene rises to 70–80% the disease complex—often involving both the nervous system and muscles such as the heart—may suddenly appear [74,75]. This level of homoplasmy presumably represents the break point beyond which metabolic compensation is impossible.

In humans with iron overload, iron-mediated damage to mtDNA in an organ such as the heart—assuming that it does occur—may take a period of years but the consequences can become manifest precipitously. In fact, Liu and Olivieri [5] remark on the frequent observation—in thalassemic patients with secondary iron overload—of a rapid deterioration of systolic function, leading to heart failure and death, which may happen within a few days (with an ejection fraction of > 50% falling to < 30% over this brief period). There could, of course, be several interpretations of such a cataclysmic event but one possibility is acute mitochondrial failure arising from years of damage to the mtDNA (similar to the drift towards homoplasy in disorders such as MERRF and MELAS mentioned above). Furthermore, Westra et al. [76] have reported on a case of hereditary hemochromatosis in which the iron-induced congestive heart failure was not reversed despite successful chelation therapy, raising the possibility that some kind of irreversible (mitochondrial?) damage had occurred. In the case of chronic iron overload, there is the additional possibility that mitochondria with sufficiently damaged genomes may preferentially replicate [77] and/or become polluting factories for the production of increased amounts of activated oxygen species which, in turn, further accelerate organ damage [78].

In aging animals and animals to which cardiotoxic anthracycline drugs have been administered, the mitochondria variably show decreased respiratory capacity, enhanced activated oxygen production, mitochondrial enlargement, reduced membrane potential, and oxidation/inactivation of the mitochondrial enzyme, aconitase [49,79,80–83], and DNA damage [62–65,84]. Certain of these features are reminiscent of mitochondrial abnormalities seen in experimental iron overload and in Friedreich ataxia [61].

Finally, there are even links, albeit indirect, to an importance of iron accumulation in the general process of aging. For example, the well-known prolongation of life span by dietary restriction (reviewed in [85]) is accompanied by decreased age-dependent iron accumulation in rats [86] and probably other species. Furthermore, inhibition of iron absorption in Drosophila melanogaster prolongs life expectancy by > 20% [87]. Similar effects on life span were exerted in house flies by administration of the chelator diethyldithiocarbamate [88]. From this perspective, some of the pathologic effects of iron overload may simply represent an accelerated form of aging.

MIGHT PEROXIDATIVE DESTABILIZATION OF LYSOSOMAL MEMBRANES CAUSE ORGAN DYSFUNCTION?

An alternative—but not mutually exclusive—mechanism for the toxic effects of iron on cells and organs involves the proposition that an accumulation of iron within the cellular lysosomal compartment will sensitize the lysosomes to damage and rupture, with release of damaging lysosomal digestive enzymes into the cytoplasm of the cell. Minimal release of lysosomal enzymes may induce transient reparative autophagocytosis, while moderate lysosomal rupture is followed by apoptosis, including caspase activation. Severe oxidative stress, resulting in massive lysosomal breakdown, is associated with necrosis (see, e.g., [89–92]). The potential importance of lysosomal instability in iron overload also has been suggested by others (e.g., [53]) and iron-mediated lysosomal instability and enhanced lipid peroxidation do occur in animal models of iron overload [93]. In vitro experiments also indicate that iron loading leads to increased fragility of lysosomal membranes [53,94], a proposition further supported by early observations made on hepatic biopsy material from hemochromatotic patients [95]. In fact, Stal et al. [96] argue that the changes in hepatic lysosomal volume density in hereditary hemochromatosis correlate very well with the extent of iron overload and are effectively reversed upon iron removal. However, it is not established whether, in vivo, these lysosomal abnormalities are an important pathophysiologic factor.

In summary, although iron overload causes a number of serious and life-threatening pathologies, the molecular bases of these unhappy events are still unknown. We have argued here that these pathologies may arise from interactions between iron and mitochondrial respiration.
If so, further knowledge of these interactions may lead to the rational design of more effective agents for the neutralization and removal of iron from patients with hemochromatosis.

Acknowledgements — The authors gratefully acknowledge grant support from the National Institutes of Health (RO1 DK 58882) and the American Diabetes Association. J.W.E. is supported by The Commonwealth of Kentucky Research Challenge Trust Fund.

REFERENCES


