Iron and Cardiovascular Dysfunction: Mechanisms and Therapeutic Implications

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It has been recognized for over three decades that tissue hypoperfusion is associated with the appearance of increased levels of iron in the plasma.[1,2] Experimental observations have documented the liberation of iron into the circulation following reperfusion of ischemic myocardium[3] and small intestine,[4] and into the urine following renal ischemia-reperfusion.[5] Similarly, we have recently demonstrated that iron is delocalized during ischemia of skeletal muscle, via a process which persists upon reperfusion.[6] Other studies have demonstrated delocalization of iron in the parenchyma of postischemic brain,[7,8] myocardium,[9] and kidney.[10]

Evidence documenting the participation of iron in the pathogenesis of tissue injury following ischemia-reperfusion is available from experiments performed in a variety of disparate organ systems, including heart,[11-13] brain,[14] liver,[15] intestine,[16] and skeletal muscle,[17,18] utilizing deferoxamine as well as other iron-chelating agents. In addition, several reports have documented lack of efficacy with the iron-loaded compound ferrioxamine,[11,12,17] and a protective effect similar to that of deferoxamine using the chemically dissimilar iron-binding compound apotransferrin has been demonstrated.[17] These data strongly suggest that the primary protective mode of action of deferoxamine is due to its ability to bind iron. Therefore, catalytically active (and chelatable) iron occupies a unique and important role in the pathogenesis of tissue injury accompanying ischemia and reperfusion.

Of course, it is now well recognized that the central events resulting in cellular injury following periods of tissue ischemia occur primarily upon reoxygenation. This injury is inflammatory in nature and is mediated in large part through the generation of reactive and toxic metabolites of oxygen. Indeed, reperfusion with an oxygenated reperfusate is required to produce tissue injury, while reperfusion with anoxic reperfusate produces very little dam-

age.[19] It is the combination of ischemia and reperfusion which generates a systemic inflammatory response, as ischemia alone, even for prolonged periods of time, does not result in significant injury in the absence of subsequent reperfusion.[20]

ROLE OF IRON IN OXIDATIVE INJURY

One mechanism through which iron is thought to participate in injury to reoxygenated tissues is via catalysis of hydroxyl radical ($OH^{\cdot}$) formation by the Haber–Weiss reaction (Fig. 1).

In this schema, iron functions as both a redox reagent, accepting electrons from superoxide anion ($O_2^{-\cdot}$), and as a Fenton catalyst, promoting the formation of hydroxyl radical ($OH^{-\cdot}$) from hydrogen peroxide (H_2O_2). Hydroxyl radical is an extremely reactive oxygen metabolite capable of initiating chain reactions of membrane phospholipid peroxidation, and is generally believed to be the free radical species most responsible for tissue damage. Quantifiable products of lipid peroxidation include conjugated dienes and malondialdehyde, also referred to as thiobarbituric acid-reactive substances.[21] In the absence of a transition metal catalyst, the Haber–Weiss reaction proceeds too slowly to be of physiologic significance, thus suggesting a major role of ferrous iron in promoting tissue injury through this pathway. In addition to catalyzing $OH^{\cdot}$ generation, iron may also react with lipid hydroperoxides formed during lipid peroxidation, leading to formation of alkoxy and peroxy radicals which are themselves capable of propagating lipid peroxidation reactions. Iron is also an efficient catalyst of tyrosine nitration by peroxynitrite, and in contrast to the Haber–Weiss reaction, catalysis of tyrosine nitration does not depend upon prior reduction of iron to the ferrous (Fe^{2+}) state.[22] Peroxynitrite (ONOO-) is a potent oxidant formed at near diffusion limited rates by the reaction of nitric oxide and $O_2^{-\cdot}$. Thus, there are multiple pathways through which delocalized iron may participate in the generation of tissue injury at reperfusion,[23] including pathways which may be independent of $OH^{\cdot}$ and peroxynitrite.[24] Indeed, it is probable that $OH^{\cdot}$ generation represents but one mechanism of iron-catalyzed oxidant injury, with final common pathways likely being multiple and dissimilar in nature. We have recently reviewed in detail the many and varied roles of iron in ischemia-reperfusion injury.[25]

$$Fe^{3+} + O2^{-\cdot} \longrightarrow Fe^{2+} + O_2$$

$$2O_2^{-\cdot} + 2H^{+} \longrightarrow H_2O_2 + O_2$$

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

$$3O_2^{-\cdot} + 2H^{+} \longrightarrow 2O_2 + OH^{\cdot} + OH^{-}(net)$$

Figure 1. Possible mechanism through which iron participates in injury to reoxygenated tissues is via catalysis of hydroxyl radical ($OH^{\cdot}$) formation by the Haber-Weiss reaction.

IRON METABOLISM AND DELOCALIZATION

The body of the average adult male contains 4 to 5 mg of iron and absorbs approximately 1 mg daily, mostly from within the proximal small intestine. A

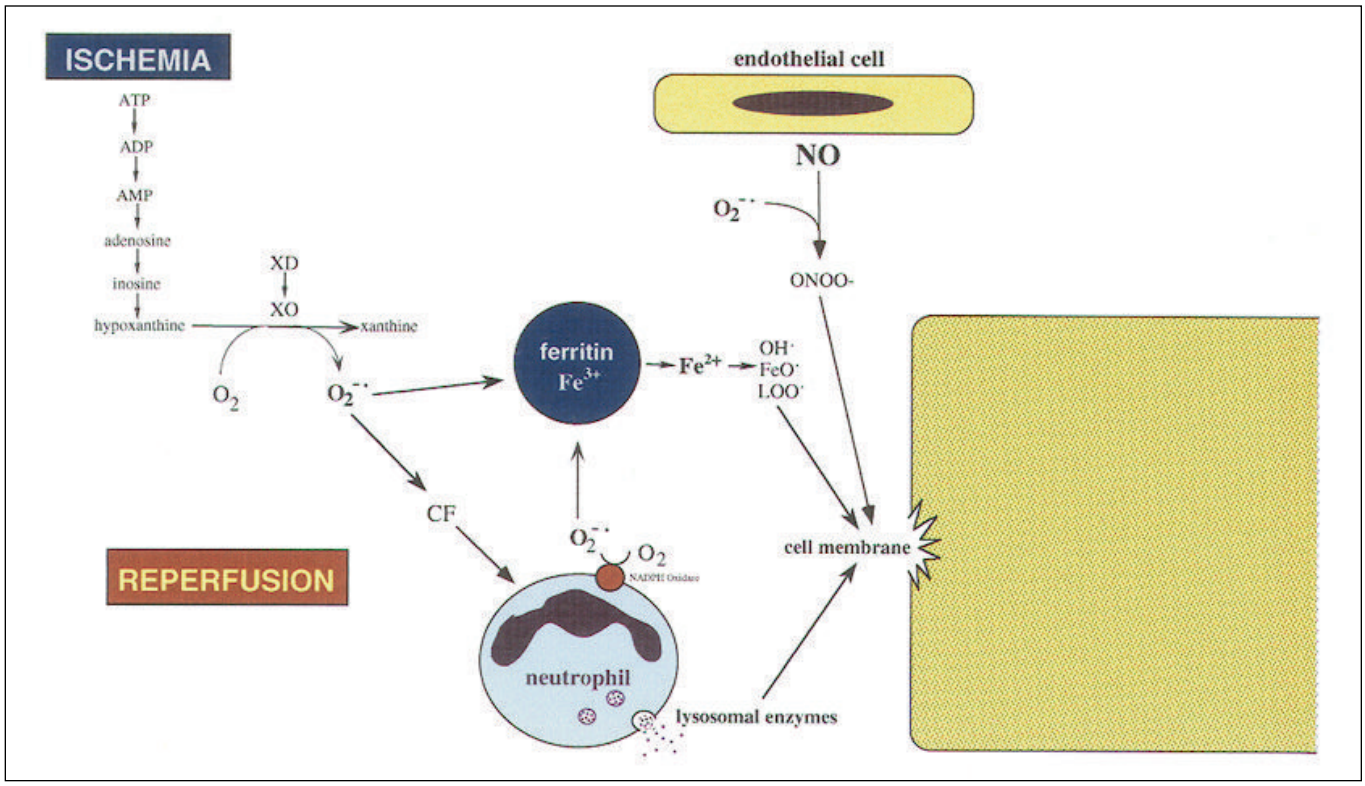

Figure 2. Proposed mechanism of neutrophil-dependent, iron-catalyzed membrane injury in postischemic tissues. XD = xanthine dehydrogenase; XO = xanthine oxidase; $O_2^{-\cdot}$ = superoxide radical; $OH^{\cdot}$ = hydroxyl radical; $FeO^{\cdot}$ = iron-oxygen complexes; $LOO^{\cdot}$ = peroxy radical; CF = chemotactic factors; ONOO- = peroxynitrite anion; NO = nitric oxide.

minor portion of the iron that is absorbed is stored within the intestinal mucosa, but the vast majority is transported to the plasma, where it is bound with high affinity to an iron-specific carrier protein, transferrin. At neutral pH, iron-loaded transferrin is a poor catalyst of free radical formation.[26] However, as the pH falls below 6, iron becomes more readily detachable from transferrin, an observation that may account for the ability of transferrin to stimulate free radical production and lipid peroxidation in acidic in vitro systems.[26,27] Although a significant degree of acidosis may be achieved in some ischemic tissues, it is unlikely that transferrin functions as an important physiologic catalyst of free radical formation.[28]

Under normal conditions, iron bound to transferrin is transported to iron-requiring cells throughout the body, predominantly erythroid precursors in the bone marrow. The iron-transferrin complex is then taken up at the cell membrane through a process of receptor-mediated endocytosis, forming a vacuole within the cytoplasm.[29] The contents of this vacuole are acidified by the action of a proton pump, resulting in the dissociation of iron from transferrin with the resultant formation of a mobile intracellular iron pool. The precise biochemical nature of this non–protein-bound iron is poorly characterized, but it is believed to consist mainly of ferrous (Fe^{2+}) iron bound to low molecular weight chelators such as phosphate esters and organic acids.

Most of the iron delivered to cells is destined for use in the synthesis of iron-containing proteins, such as hemoglobin. The majority of iron not utilized in protein synthesis is stored throughout the body as soluble ferritin and, to a much lesser extent, as the insoluble complex hemosiderin.[30] Of these storage forms, the former is perhaps the most important endogenous source of catalytic iron. Present in all types of mammalian cells studied, ferritin is comprised of 24 protein subunits arranged as a hollow sphere. The interior of the molecule communicates with the exterior by way of six narrow (3-4 A) channels.[31] Each molecule is capable of storing up to 4500 iron atoms, mainly as ferric oxyhydroxide and ferric oxyphosphate. Mobilization of iron from within the ferritin core requires its reduction to the ferrous state in the presence of low molecular weight iron chelators.[28,32]

IRON AND REPERFUSION INJURY

Evidence supporting the role of iron in reoxygenation injury is provided by studies in which the administration of agents that bind iron is shown to limit the free radical production and cellular dysfunction otherwise seen following reperfusion. Of these agents, the most widely utilized in experimental models is the iron chelator deferoxamine (DFO), a bacterial siderophore derived from the species *Streptomyces pilosus*. DFO is currently employed in the treatment of acute and chronic iron toxicity and has recently been shown to protect against oxidative injury in a number of organ systems. Although DFO may itself act as a free radical scavenger,[28] it has now been established with a reasonable degree of certainty that its protective effect in experimental models is a direct result of its ability to bind free iron.[16,17,33]

There is substantial evidence that oxygen-derived free radicals are produced in abundance following the reperfusion of ischemic myocardium[34] and during operations requiring cardiopulmonary bypass.[35,36] In several animal models of myocardial ischemia and reperfusion, DFO has been shown to reduce free radical production and to improve postischemic ventricular functional and metabolic recovery.[13,37-42] Reddy and colleagues have demonstrated a reduction in infarct size when DFO is given prior to the onset of myocardial ischemia, but no such protective effect when the drug is given immediately before the onset of reperfusion.[43] Contrary to these results, Badylak and coworkers have reported that the administration of DFO prior to reperfusion of the ischemic rat heart reduces myocardial enzyme release and reduces postischemic coronary vascular resistance.[44] Histologic evaluation of hearts in the DFO-treated group revealed a preservation of cell membrane integrity not seen in untreated controls. In a dog model of coronary revascularization, Illes and colleagues noted a diminution in myocardial "stunning" when DFO was added to the cardioplegic solution, suggesting that iron-catalyzed free radical formation may contribute to myocardial dysfunction following coronary artery bypass.[45] It is likely that oxygen free radical-mediated injury also occurs in the setting of cardiac transplantation when the stored, ischemic donor heart is reoxygenated with the donor's blood. The role of iron in this setting has been studied by Menasche and coworkers,[46] who have demonstrated that the addition of DFO to the cardioplegic solution in rats results in improved ventricular performance and coronary flow in donor hearts following reperfusion. As DFO has no inotropic or rheologic effects, this protection likely results from a decrease in the amount of free iron available to catalyze free radical formation and lipid peroxidation.

The kidney, often subjected to substantial periods of ischemia during shock and renal transplantation, is another potential target for injury wrought by oxygen-derived free radicals. Here, as in the heart, the administration of iron chelators prior to reoxygenation has been associated with significant protection against oxidative damage. In studies involving up to two hours of warm renal ischemia, pretreatment with DFO has been shown to result in a dose-related decrease in the formation of lipid peroxidation end-products following reperfusion.[47] A significant reduction in lipid peroxidation has also been documented in donor kidneys flushed with DFO-containing solutions prior to ischemic cryopreservation.[48] Pretreatment with DFO results in a similar reduction in oxidative injury in reperfused renal transplants subjected to 24 hours of cold ischemia.[49] The clinical significance of these findings is underscored by the findings of Baron and coworkers in a study of canine renal autografts subjected to 30 minutes of warm renal ischemia and 48 hours of machine perfusion.[50] Here, the addition of DFO to the perfusate was found to result in a significant reduction in peak serum creatinine and an overall improvement in survival, suggesting that iron-catalyzed oxidative injury is a critical mediator of postischemic renal dysfunction.

The small bowel is particularly vulnerable to free radical-mediated injury, perhaps a result of the relatively high levels of xanthine dehydrogenase present in the gastrointestinal mucosa. As in other organ systems, pretreatment with iron chelators has been shown to be of benefit in models of small intestinal ischemia and reoxygenation. In a feline model of high-grade partial

mesenteric ischemia, for example, the administration of DFO reduced the formation of lipid peroxides as effectively as SOD and catalase together or allopurinol alone.[51] Hernandez and coworkers observed that the infusion of DFO five minutes prior to reperfusion significantly attenuated postischemic microvascular injury in isolated ileal segments.[16] Similar protection was afforded by infusion of the iron-binding protein apotransferrin, but not by the administration of iron-saturated DFO, thus suggesting that the presence of "free" chelatable iron is essential to the development of postischemic injury in the small intestine.

In contrast to other tissues in which ischemia of relatively short duration may have catastrophic consequences, skeletal muscle is able to withstand comparatively prolonged periods of ischemia. This tolerance most likely results from the relatively low resting metabolic rate of skeletal muscle, as well as from the presence of low oxidative fiber types and alternate energy substrates such as phosphocreatine and glycogen.[52] In addition, skeletal muscle possesses a remarkable regenerative capacity not shared by many other organ systems and can regain a significant degree of contractile function even after suffering ischemic necrosis.[52] Nevertheless, reoxygenation of ischemic skeletal muscle has been shown to result in significant contractile dysfunction and irreversible myonecrosis,[53] as well as in considerable microvascular injury, an important factor in the development of the compartment syndrome.[54] In addition, acute revascularization of postischemic skeletal muscle has been associated with potentially life-threatening structural and functional derangements in remote organ systems, including pulmonary microvascular injury[55-58] and myocardial depression.[59] Iron has been shown to play an important, if not critical, role in the progression of reperfusion-associated tissue injury in this setting. In a rat model of hindlimb ischemia, Smith and colleagues[17] observed that pretreatment with DFO mitigated the rise in capillary permeability previously documented in reoxygenated skeletal muscle, whereas pretreatment with iron-saturated DFO did not. This finding was later supported by the work of Perler and coworkers[60] who found that the administration of DFO to rabbits subjected to seven hours of femoral artery occlusion significantly attenuated the rise in anterior compartment pressure measured following reperfusion. Data from our laboratory also supports the salutary effects of iron binding upon reperfusion of postischemic skeletal muscle, in a rat model of high-grade partial hindlimb ischemia.[18] In these experiments, an ischemic interval of 90 minutes was accompanied by peroxidation of membrane phospholipids, as measured by muscle malondialdehyde content, and membrane dysfunction, as indicated by depolarization of resting transmembrane potential (E_m). Upon reperfusion, muscle malondialdehyde content continued to increase, while the muscle cell membrane remained depolarized. Pretreatment with DFO prior to ischemia, however, prevented lipid peroxidation and permitted partial repolarization of the cell membrane upon reperfusion.

The administration of DFO has been shown to protect against oxidative reperfusion injury in several other tissues as well, including stomach,[61] liver,[15] lung,[62] and skin.[63] In fact, the beneficial effects of iron chelation have now been demonstrated in nearly every organ system in which oxygen free radical-mediated reperfusion injury has been described. This observation, along with the abundance of data implicating the neutrophil as an important mediator of reoxygenation injury, suggests that the organ dysfunction that frequently follows the sequence of ischemia-reperfusion occurs via a leukocyte-dependent, iron-catalyzed process of free radical formation and membrane lipid peroxidation (Fig. 2).

THERAPEUTIC ASPECTS OF IRON BINDING.

A fundamental event in the pathophysiology of ischemia-reperfusion is a burst of free radical formation occurring almost immediately upon the reestablishment of blood flow.[34,64] As a result, any therapeutic intervention aimed at effectively limiting oxidative injury must be initiated prior to, or immediately upon, reperfusion. Limiting the availability of catalytic iron through the use of iron chelators provides a means by which to interrupt the biochemical pathways that produce potentially damaging oxidant species. However, widespread clinical use of DFO in the setting of ischemia-reperfusion is precluded by its rapid excretion and toxic systemic side effects, the most significant of which is a tendency to induce hypotension and myocardial depression following high-dose intravenous administration.[65-67] Efforts to prolong the half-life and limit the toxicity of DFO have led to the synthesis of several macromolecular forms of the drug through the covalent attachment of DFO to high molecular weight polymers, such as hydroxyethyl starch and dextran.[65,68] These DFO conjugates (Biomedical Frontiers, Minneapolis, Minn.) bind iron with the same high affinity and specificity of the native compound and retain the ability to inhibit iron-catalyzed lipid peroxidation despite their inability to enter the intracellular space.[68] These compounds offer several advantages over the unconjugated drug, however, including a circulating half-life that is 10 to 30 times that of DFO alone[68] and the absence of adverse hemodynamic effects following high-dose intravenous administration.[66]

The potential utility of DFO conjugates in the prevention of oxidative reperfusion injury has recently been demonstrated in several animal models. Maruyama and coworkers have reported that the administration of hydroxyethyl starch-conjugated DFO, but not of the iron-saturated polymer, significantly improves segmental shortening following regional myocardial ischemia and reperfusion.[12] The protective effects of synthetic DFO conjugates have also been documented by us in the rat model described above, wherein pretreatment with pentastarch-conjugated DFO resulted in a significant decrease in lipid peroxidation and membrane dysfunction following reoxygenation.[18] It is noteworthy that only half of the dose (30 mg/kg) of pentastarch-conjugated DFO was required to produce the same effect as free DFO (60 mg/kg) in our experiments, thus potentially limiting toxicity via effective dose reduction. Drugas and coworkers have noted that starch-conjugated DFO protects against reperfusion-induced microvascular injury following hepatic ischemia.[69] Rosenthal and coworkers have recently shown that the administration of hydroxyethyl starch-conjugated DFO prevents brain lipid peroxidation and improves neurologic recovery in rats resuscitated from cardiac arrest.[70]

Iron binding with DFO has also been shown to be effective in preventing the systemic inflammatory response accom-

panying experimental burn injury. The administration of intravenous DFO conjugated to hetastarch during the resuscitation period following standardized scald injury in sheep was associated with marked reduction in oxidative injury to the lung and liver, as assessed by measurement of lipid peroxidation by malondialdehyde.[71] In a subsequent experimental study of standardized smoke inhalation injury in sheep, these same investigators found that aerosolized DFO-pentastarch conjugate, but not free DFO, administered after inhalation injury prevented the subsequent lung and systemic inflammatory injury.[72] Thus, an important role for iron in the pulmonary and systemic inflammatory responses following thermal injury appears likely. Clinical testing of the DFO-pentastarch conjugate in this setting is currently underway.

Iron is released into the circulation during hemorrhagic shock and may contribute to subsequent cellular injury by catalyzing membrane lipid peroxidation. In the clinical setting, successful resuscitation from shock is often followed by the development of multiple organ system dysfunction, an event that may be mediated to a large extent by the reactions of oxygen-derived free radicals.[73] Iron chelation has now been shown in several models to improve survival following hemorrhagic shock. Treatment of dogs in hemorrhagic shock with DFO prior to resuscitation results in improved survival and more rapid recovery of neurologic function.[74] Griffin and Babbs have demonstrated that the hepatocellular injury observed following the restoration of intravascular volume in dogs may be attenuated by treatment with DFO prior to resuscitation.[75] In a porcine hemorrhagic shock model, Jacobs and coworkers noted improved survival, decreased hepatic lipid peroxidation, and lower serum aspartate aminotransferase (AST) levels 24 hours following resuscitation with a pentastarch-DFO conjugate as compared with that using Ringer's lactate or pentastarch alone.[76] Survival rates were also better in animals resuscitated with pentastarch-DFO when compared to those treated with solutions containing free DFO, one-third of which developed profound hypotension within minutes of the initiation of resuscitation and rapidly expired. These results support the hypothesis that reperfusion following periods of global ischemia precipitates tissue injury and organ dysfunction through a process of iron-catalyzed peroxidation of membrane lipids.

Cardiopulmonary bypass has been referred to as a state of "controlled shock," i.e., global ischemia followed by reperfusion. Menasche and colleagues have demonstrated the potential utility of iron binding in patients undergoing cardiopulmonary bypass to whom DFO was given intravenously and as an additive to the cardioplegic solution.[77] Polymorphonuclear leukocytes isolated after bypass from right atrial blood samples of DFO-treated patients were found to produce significantly fewer superoxide radicals than those of control patients, supporting the hypothesis that iron-catalyzed, leukocyte-dependent free radical production may play a central role in mediating organ dysfunction after reperfusion. In a subsequent study by the same authors, the administration of DFO to patients undergoing cardiopulmonary bypass resulted in a diminished susceptibility of circulating low-density lipoproteins to peroxidation, perhaps due to a decreased consumption of endogenous antioxidants following bypass.[78] Further evaluation of the DFO-pentastarch conjugate in this setting is ongoing.

In summary, iron-catalyzed free radical production and lipid peroxidation have been shown to cause significant parenchymal and microvascular injury following the successful restoration of blood flow to ischemic tissues. As investigations into the pathophysiology of ischemia-reperfusion injury have begun to define the complex interactions between postischemic cellular metabolism, microvascular function, and the host immune response, it has become apparent that the release of catalytic iron from storage sites during ischemia plays a critical role in the subsequent generation of oxidative tissue damage. Preclinical investigations in models of shock resuscitation, organ preservation, transplantation and vascular reconstruction, as well as the clinical investigation of cardiopulmonary bypass, have shown the potential of iron chelation as a practical and effective means of protecting against the deleterious effects of reoxygenation in postischemic tissues. The effectiveness of this and related therapeutic strategies in the clinical setting remains under active investigation. STI

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