High-dose systemic iron chelation attenuates reperfusion injury
Bo E. Hedlund and Philip E. Hallaway
Biomedical Frontiers, Inc., 1095 Tenth Ave. S.E., Minneapolis, MN 55414, U.S.A.

Introduction
This discussion will focus on two areas. The first will deal with the release and movement of iron from intracellular storage sites to the vascular compartment following an ischaemic episode. The conditions necessary for this release and the question of whether the quantities of iron involved are relevant to clinical situations occurring in acute-care medicine will also be addressed. The second area of discussion will focus on a number of preclinical studies involving the use of deferoxamine or a high-molecular mass analogue of deferoxamine in the treatment of a reversible myocardial dysfunction induced by short-term regional ischaemia. The critical role of 'free' iron in catalysing reactions leading to the formation of oxygen- and lipid-derived reactive species has been reviewed extensively by several authors [1, 2] and will not be discussed here.

Release of iron following ischaemia
The release of significant amounts of iron into the vascular compartment during severe hypotension imposed by haemorrhage was first observed by Mazur and collaborators in the mid 1950s [3]. A similar observation was made a few years later by Janoff and colleagues [4]. Each of these studies reported increases of three–fourfold in the plasma iron levels. Because of a concomitant decrease in the total iron-binding capacity (TIBC) observed in these studies, it is evident that the increased plasma iron was not due to iron-containing proteins appearing secondary to tissue damage.

Several studies dealing with isolated organs, both in vivo and ex vivo, have provided convincing evidence that iron is released from an ischaemic organ into the vascular space. In a study of the ischaemic kidney in the rat, Paller and Hedlund [5] noted a 10-fold increase in urinary iron levels following 60 min of warm ischaemia. This iron was measured by the bleomycin technique [6, 7], indicating that the iron was chemically reactive. Likewise, Robinson and Hedlund [8] observed that the plasma iron levels of rats increased by about 60% after 120 min of complete intestinal ischaemia. In contrast, the circulating iron levels of animals exposed to surgical intervention but no ischaemia decreased 20–40%. Thus, the increased level of iron observed during reperfusion more than compensates for the hypoferraemia normally occurring in response to non-ischaemic trauma and/or infection.

Voogd et al. [9] have quantitated the magnitude of the 'low-molecular-mass iron pool' in the perfused heart, and have clearly demonstrated the release of substantial quantities of chelatable iron from storage sites in the myocardium after ischaemia. Of particular importance to this discussion is the observation that the iron levels in the perfusate increased 30-fold following an ischaemic insult of 45 min, but did not increase during anaoxia. The dramatic increase in measurable iron in the ischaemic but not anoxic heart indicates that a combination of low pH and a metabolically-depleted cell is necessary for iron release. If the pH is maintained by buffering, the release of iron is largely inhibited.

Although these studies provide clear evidence that iron is released from ischaemic organs, they do not identify whether the source of this iron is predominantly endothelial or parenchymal. A question arises in this context: are the endothelial cells particularly susceptible to the effect of ischaemia, or are all cell types equally susceptible to the biochemical consequences of lack of oxygen? It might be assumed that cells that consume larger amounts of oxygen would be more sensitive to ischaemia, but these cells may also have more efficient means of protecting themselves against oxidant injury. This hypothesis, to a first approximation, can be tested in culture by exposing a variety of cells (e.g. hepatocytes, endothelial cells, myocytes and epithelial cells) to a hypoxic insult followed by reperfusion with exposure to normal atmosphere. The measurement of 'free' iron in the cell culture medium following such an experiment would provide a clue to the hypoxic sensitivity of each cell type.

Unfortunately, there are no systematic studies of differential sensitivity of cultured cells to oxidant injury nor of the amount of iron released by different cell types after an ischaemic insult. Clearly, iron can be released from epithelial cells as demonstrated by Paller and Hedlund [10] using proximal tubule epithelial cells from rats in primary culture exposed to 60 min of anoxia followed by 30 min of reoxygenation. These authors demonstrated a 67% increase in the amount of ferrozine-measurable iron

Abbreviations used: DFO, deferoxamine; FO, ferrioxamine; HES-DFO, hydroxyethyl starch-deferoxamine conjugate; LAD, left anterior descending artery.
in the cell homogenates and extracellular medium following anoxia and reoxygenation. The relationship between this iron and the total cellular iron pool was not determined.

In addition, Paller and Hedlund examined the protective effects of deferoxamine (DFO), a hydrophilic iron chelator with a $M$, of 560, and a macro-molecular form of this chelator, hydroxymethyl starch-deferoxamine conjugate (HES–DFO) [11]. Neither of these chelators, especially the macro-molecular form, can readily enter cells. Both chelators provided roughly the same degree of protection, as measured by release of lactate dehydrogenase. These studies suggest that a significant fraction of the protection observed in studies utilizing DFO and HES–DFO occurs in the extracellular compartment. The ability of hydrophilic chelators, and in particular the high-molecular-mass form of DFO, to protect cells against oxidant injury is an intriguing observation which will be discussed further in the following section.

**Diffusible versus vascular iron chelators**

In comparing the efficacy of deferoxamine with that of conjugated deferoxamine, many factors must be considered. In this section we will briefly review several studies in which DFO and/or HES–DFO have been used. The comparison will focus on studies involving the ‘stunned myocardium’, a reversible, short-term ischaemic insult to the myocardium. The most common model of the stunned myocardium involves a 15 min occlusion of the left anterior descending (LAD) artery in the dog followed by at least 2 h of reperfusion. Using this model, two groups of investigators have shown independently that deferoxamine reduces myocardial dysfunction caused by ischaemia and reperfusion.

The earliest study of the effect of DFO on reducing myocardial dysfunction following a 15 min LAD occlusion was published by Bolli et al. [12]. In this pretreatment model, DFO (10 mg/kg) was given over 45 min starting 30 min prior to ischaemia and continuing for the entire 15 min of occlusion. Another 7 mg/kg was administered during the 4 h of reperfusion. This dose may have been rather low, but the authors noted that higher doses were associated with hypotension. Dogs treated with the chelator showed an improvement of 8–12% in the systolic thickening fraction, a direct measure of post-occlusion functional recovery, as compared to the saline-treated control group. The improvement was statistically significant at all time points during reperfusion. For comparison to other studies, the observed improvement in thickening fraction corresponds to a 30–45% improvement in return of function as compared to the saline control group (authors' estimates).

In a similar study, Farber et al. [13] administered DFO intra-atrially at a dose of 500 mg (18–26 mg/kg depending on the animal's weight) to dogs subjected to 15 min of LAD occlusion. Infusion of the drug was initiated 15 min prior to LAD occlusion and was continued throughout the ischaemic period. Thus, the intra-atrial dose used by these authors was slightly higher than the intravenous dose given by Bolli et al. Furthermore, Bolli et al. continued slow infusion during reperfusion while Farber et al. discontinued administration at the time of reperfusion. The net recovery in thickening fraction in the Farber study is equivalent to a 35–50% improvement over the saline-treated controls (estimates made by these authors), which is remarkably close to the improvement observed by Bolli's group. It should be noted that Farber et al. used implanted piezoelectric crystals rather than a Doppler probe to quantitate thickening fraction.

In a more recent study, Bolli's group [14] further evaluated the ability of DFO to improve post-stunning function in the canine myocardium. In this case, Bolli used intracoronary infusion of the drug, presumably to maximize the protective effect of the drug on the myocardial vasculature. To assess when chelator therapy was most efficacious, the authors compared the effect of infusing DFO at 3 mg/kg either immediately before or immediately after initiation of reperfusion, with low-dose infusions continued throughout the reperfusion period. In addition, Bolli et al. compared the myocardial function in these animals with animals receiving either ferrioxamine (FO; iron-saturated DFO) or saline. The group observed a remarkable recovery of function in the animals receiving DFO before, but not after, the initiation of reperfusion. The recovery of contractile function amounted to between 80 and 90%, nearly twice as large as the improvement noted in the two previous studies. There was no significant improvement in the animals receiving DFO one minute after initiation of reperfusion, nor in animals receiving FO. The authors further demonstrated, using spin-trap techniques, that a burst of reactive molecules appeared in the coronary perfusate at reperfusion and that the amount of these reactive compounds was greatly reduced in animals receiving DFO prior to reperfusion. The primary conclusion that can be drawn from this study is that the vascular concentration of
DFO at the beginning of reperfusion is of critical importance in determining the efficacy of the drug. Obviously, intracoronary infusion is not always available as a means of delivering drugs in acute cardiac care. Maruyama et al. [15] have compared the ability of DFO and hydroxyethyl starch-DFO conjugate (HES-DFO) [11] to protect the stunned myocardium when given intravenously in a pretreatment mode. Animals were treated with HES-DFO, the iron-saturated form of the chelator (HES-FO), native DFO or saline. All treatment groups received 50 mg/kg of DFO or FO equivalents over a 45 min period starting 30 min before ischaemia. In this study, only animals receiving HES-DFO had a significantly improved cardiac function compared with the control, saline-treated animals. The degree of protection averaged about 40% during the reperfusion. The lack of protection seen with free DFO is somewhat surprising in light of prior studies, especially Bolli's study using intra-atrial infusion. The lack of efficacy of the free drug may be due to the inherent toxicity of DFO administered intravenously at high doses. Bolli et al. noted a statistically significant decrease in the blood pressure during infusion of the DFO.

The haemodynamic effects of intra-atrial infusion of DFO and HES-DFO have been examined by Forder et al. [16] in conscious, instrumented dogs. This study may be more relevant to the clinical setting in which patients are treated without anesthesia. These investigators noted hypotension and tachycardia following intra-atrial infusion of 50 mg/kg of DFO over 15 min. In addition, a direct effect on cardiac function, which manifested itself as a decrease in thickening fraction, was noted during the infusion period. Thus, changes in cardiac function were observed in the absence of an ischaemic insult, or simply by the intra-atrial infusion of DFO (3.3 mg/kg/min). Whether or not the cardiotoxic effect of high concentrations of DFO accounts for the observed lack of efficacy of DFO in the study by Maruyama et al. is uncertain, but the use of free DFO in cardiac medicine must be approached with a great deal of caution.

In a related study, Mousa and coworkers [17] evaluated the relative efficacies of DFO and HES-DFO in a porcine model of myocardial ischaemia. The group demonstrated that 25 mg/kg of chelator given intravenously 10 min into the 20 min ischaemic period resulted in a significant improvement in contractile function. Both forms of the chelator also decreased the amount of tissue oedema as measured by wet-dry weight ratio, with the conjugated form showing the greatest protection. Pigs treated with DFO at a dose of 25 mg/kg exhibited significant hypotension during the initial period of reperfusion.

The preclinical studies using DFO and related compounds in the stunned myocardium can be summarized as follows. (i) Iron chelation therapy using deferoxamine attenuates myocardial dysfunction following short-term ischaemia and subsequent reperfusion. (ii) The most effective protection is obtained when the therapeutic agent is administered directly to the compromised microvascular, i.e. by intracoronary infusion. (iii) The therapeutic agent has to be present in circulation at the time of reperfusion in order to be effective. (iv) When systemic administration is used, the high-molecular-mass form of DFO appears to provide better protection than the parent drug.

Systemic iron chelation appears to offer a viable means of decreasing the magnitude of radical-mediated injury occurring both during and after ischaemia. Deferoxamine, the only clinically acceptable iron chelator, has been used in a multitude of preclinical studies of the ischaemic myocardium, often showing efficacy in reducing reperfusion injury. However, the use of this drug in clinical medicine is hampered by its tendency to induce hypotension when administered intravenously. Attachment of deferoxamine to polymers [11] such as hydroxyethyl starch yields high-molecular-mass chelators that lack the toxicity associated with the parent drug. These compounds, unlike the free drug, remain in the vascular compartment and have proved efficacious in several preclinical models involving radical-mediated tissue injury following ischaemia and reperfusion. The ability of a high-molecular-mass iron chelator to reduce reperfusion injury suggests that a significant component of this injury involves the microvasculature, in particular the endothelial lining of small blood vessels.

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Composition and organization of the NADPH oxidase of phagocytes and other cells

O. T. G. Jones, S. A. Jones, J. T. Hancock and N. Topley*

Department of Biochemistry, School of Medical Sciences, University of Bristol, Bristol BS8 1TD and *Institute of Renal Medicine, KRUF, Cardiff Royal Infirmary, Newport Road, Cardiff CF2 1SZ, U.K.

Introduction

Superoxide ($O_2^-$) is produced by phagocytic leukocytes in response to a variety of stimuli. The activation of $O_2^-$ production by neutrophils following binding by plasma membrane receptors of the phagocyte to opsonized bacteria or to peptides from degraded bacteria is of great importance. The $O_2^-$ and its reaction products contribute to the microbicidal activities of these cells, and so resistance to infection. In the rare genetic disease, chronic granulomatous disease (CGD), neutrophils lack one or more of the protein components of the $O_2^-$-generating oxidase and patients suffer repeated infections, demonstrating the involvement of the oxidase in combating infection [1, 1a].

The oxidase that is located in the plasma membrane uses cytosolic NADPH as electron donor and so is known as the NADPH oxidase. It contains a low potential cytochrome $b$ and FAD [2]. Comparison of its sequence with that of other flavoproteins suggests that the FAD-binding site may be on the cytochrome $b$ itself [3]. The cytochrome $b$ is a heterodimer with subunits with $M_s$ of approx. 91 kDa ($\beta$ subunit) and 22 kDa ($\alpha$ subunit) [4, 4a]. The low oxidation/reduction potential of the cytochrome $b$ (Em$_{pr} = -245$ mV) is suited to its function in an electron transport system linked to the reduction of $O_2$ to $O_2^-$ (Em$_{red} = -160$ mV; [5]), and it is kinetically competent to act as the direct donor to $O_2$ [6]. A combination of steady-state spectra and kinetic evidence suggests that every electron passing to $O_2$ must be transferred through cytochrome $b$ [6]. The simplest arrangement of these components is in a linear sequence:

$$\text{NADPH} \rightarrow \text{FAD} \rightarrow 2\text{Cyt}$$.b $\rightarrow 2\text{O}_2 \rightarrow 2\text{O}_2^-$.

(1)

An active NADPH oxidase can be assembled from neutrophil plasma membranes by the addition of cytosol together with either arachidonic acid or SDS [7]. Three cytosolic protein components are required for formation of the oxidase complex and these have been purified and sequenced. Two of the proteins are specific for the oxidase — a 47 kDa protein ($p47$-phox) and a 67 kDa protein ($p67$-phox) and the third is a small (21 kDa) G-protein of the ras family [8]. A mixture containing purified cytochrome $b$, added FAD and SDS and pure recombinant $p47$-phox, $p67$-phox and $p21$ ras1 catalysed high rates of superoxide production when NADPH was added [9]. These proteins form the apparent minimal requirements for an active NADPH oxidase, as shown in Figure 1. The sequence of each of these proteins has been established and it is possible to probe for their presence in a variety of cells (see below) using Western blots and Northern and Southern blotting.

Received 4 January 1993