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SUPEROXIDE RELEASE BY HUMAN POLYMORPHONUCLEAR LEUKOCYTES IN THE PRESENCE OF DEFEROXAMINE

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ABSTRACT

Background and Objective. Anecdotal reports in patients with acute and chronic iron overload have recently indicated that the efficacy and safety of an alternative chelation program including intravenous and/or continuous delivery of deferoxamine (DFO) may be in contrast with the risk of developing lung injury. Production of oxygen radicals has been postulated to be an important mechanism by which polymorphonuclear leukocytes (PMNs) could cause tissue injury in patients undergoing this alternative treatment method.

Methods. PMNs obtained from healthy donors were incubated at 37°C for 30 min with DFO (across the drug concentration 0.125 to 10 mg/mL). Superoxide (O_2^-) production was measured by superoxide inhibitable cytochrome *c* reduction as well as by an NBT densitometric kinetic test. In the same run the effect of lipid peroxidation was demonstrated by means of a malonyldialdheyde (MDA) assay.

Results. Preincubation of PMNs with any study concentration of DFO significantly enhanced O_2^- release as well as MDA production upon PMA stimulation. Maximal intracellular and extracellular O_2^- release as well as MDA production occurred at certain drug concentrations.

Interpretation and Conclusions. Our *in vitro* findings suggest that O_2^- release may be an additional detrimental contribution to tissue injury in some patients who develop pulmonary toxic effects while on intravenous and/or continuous DFO administration.

Key words: reactive oxygen species, NBT, superoxide ion, cytochrome c reduction

Deferoxamine (DFO), a trihydroxamate siderophore derived from *Streptomyces pilosus*, is currently the most clinically useful iron-chelating agent available. Since its introduction in the 1960s[1] DFO has been extensively used for chelation therapy in iron overloaded states. The chelating drug DFO, when administered regularly via subcutaneous infusions to patients with iron overload, removes excess liver iron, helps prevent iron-induced cardiac disease, and improves survival.

Nonetheless, not all patients realize these benefits. Some who are poorly compliant with standard chelation therapy may continue to accumulate iron and later die of hemochromatosis. Cardiac function in patients with massive iron burden or preexisting iron-induced heart disease may deteriorate despite standard subcutaneous DFO infusions. Consequently, alternative chelation programs including intravenous delivery of DFO have been used for patients who do not comply with conventional chelation therapy or who have excessive iron stores or established iron-induced organ disease when chelation therapy is begun. In fact, this alternative method of chelation therapy has been shown to result in greater iron excretion than can be obtained with conventional subcutaneous treatment.[2,3] Moreover, intravenous infusion is free of the local side effects that commonly occur at the site of subcutaneous infusion. An additional approach to this alternative chelation program has included intensive, continuous (for more than 24 hours) treatment with intravenous DFO infusions for patients with acute and chronic iron overload.

Recently, the potential usefulness of DFO as an antiproliferative, anti-inflammatory, and immunosuppressive agent has inspired an interest in treating empirically patients with solid tissue tumors with intravenous and/or continuous infusions of DFO.[4-6] However, anecdotal reports have recently indicated that the efficacy and safety of this alternative chelation therapy may be in contrast with the risk of developing lung injury. Interestingly, patients with pulmonary toxic effects following this program met clinical, physiological and, in some instances, necropsy criteria for the diagnosis of adult respiratory distress syndrome (ARDS).[7-9]

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Although the precise pathophysiology of the pulmonary syndrome[10-13] was not clear from the reported cases, it was postulated that DFO might cause damage in the lungs by the production of free radicals. These observations needed to be extended and it was with this aim that we undertook *in vitro* studies on O_2^- release by PMNs in the presence of DFO.

Materials and Methods

Reagents

The following reagents were employed: deferoxamine B methanesulfonate (Desferal CIBA-GEIGY, Basel, Switzerland). Dulbecco's phosphate buffer (without calcium chloride and magnesium chloride) pH 7.4. Phorbol myristate acetate (PMA) 1 µg/mL in Dulbecco's phosphate buffer (Sigma Chemical Co., St.Louis, MO, USA); nitroblue tetrazolium (NBT) 0.2% in Dulbecco's phosphate buffer (Sigma); N-formil-leucyl-phenylalanine (fMLP) 10^{-7} mol/L (Sigma); histopaque 1077 and 1119 (Sigma); EDTA K3 (Sclavo Diagnostics); cytochrome *c* from horse heart 150 nmol/mL (Sigma); superoxide dismutase (SOD) (EC 1.15.1.1) from horseradish 40 µg/mL (Sigma), Hank's balanced salt solution (Flow); $FeCl_3$-$6H_2O$ (Merck, Darmstadt, Germany).

Isolation of PMNs

PMNs obtained in EDTA K3 from phlebotomized healthy donors were purified by a double gradient technique.[14,15] Cells were assessed for 95% viability by trypan blue exclusion.

Pretreatment of PMNs with deferoxamine

PMNs were suspended in Dulbecco's phosphate buffer at the final concentration of 10^7 cells/mL in the presence of different concentrations of DFO (see below) and incubated in polypropylene vials for 30 min at 37°C in air containing 5% CO_2 in a CO_2 incubator.[16] Cells were then washed twice in Dulbecco's phosphate buffer and resuspended before testing.

For the analyses of the effect of iron-saturated DFO, 100 µL $FeCl_3$ (2M) was mixed with 4 mL DFO (50 mM). The mixture were adjusted first to pH 2 (with 6N HCl) and then, under continuous stirring, to pH 7.4 (with solid $NaHCO_3$), with a final volume of 5.0 mL.

Evaluation of superoxide release by PMNs

Production of intracellular O_2^- was assayed densitometrically by nitroblue tetrazolium densitometric kinetic test (NBT DK test) as previously described.[17,18] A 250 µL suspension containing a final concentration of 5×10^5 cells in Dulbecco's phosphate buffer was placed, under continuous stirring at a constant speed of 500 rpm and under incubation at 37°C, in an aggregometer (Aggrecorder II Daiichi Kagaku Co., Japan). Percentage of transmittance (T%) was evaluated by means of a diode (LED) with a maximum wavelength of 650 nm as light source. In order to evidence the rapidly increasing T% to 65%, a mixture of 500 µL, including 250 µL of 0.2% NBT in Dulbecco's buffer and 250 µL of either 1 µg/mL PMA or fMLP 10^{-7} mol/L in the same buffer, was added just one minute after the measurement was started. NBT reduction affecting T% was evaluated for a total of 25 minutes.

Extracellular O_2^- production was assessed spectrophotometrically by means of superoxide dismutase inhibitable cytochrome *c* reduction, and a continuous assay was performed in a double wavelength spectrophotometer (Beckman DU 65) equipped with thermostated cuvette holder (37°C).[19] Briefly, cells were suspended in Hank's balanced salt solutions, pH 7.4, before they were added to 1 mL cuvettes containing 80 µmol/L cytochrome *c* with a final volume and concentration of 0.995 mL and 5×10^5 cells, respectively. Reduction of cytochrome *c* was measured at 550 nm with a reference wavelength of 540 nm. Either fMLP (10^{-7} mol/L) or PMA (1 µg/mL) was added to the reaction mixture in a final volume of 1 mL. The time course of cytochrome *c* reduction was followed on the recorder built into the spectrophotometer. Extracellular O_2^- production was calculated as nmoles for 5×10^5 PMNs.

Determination of lipid peroxidation

Lipid peroxidation was tested spectrophotometrically by means of malonyldialdheyde (MDA) assay including the thiobarbituric acid reaction.[20] Briefly, cell stimulation was achieved by adding PMA (final concentration 1 µg/mL) to PMNs preincubated with DFO. The reaction was stopped after 4 minutes with the addition of 20% (wt/vol) trichloroacetic acid (1.2 mol/L). MDA concentration was calculated as nmoles for 5×10^5 PMNs.

Statistical analysis

Analysis of variance (ANOVA) with Bonferroni multiple comparison tests was performed. All statistical tests were based on a significance level of less than 0.05.

Results

In experiments on the effects of DFO alone on resting PMNs, the results indicated that all study concentration across the range from 0.125 mg/mL to 10 mg/mL failed to activate the respiratory burst.

Effects of iron-saturated DFO on PMNs upon PMA or fMLP stimulation

Neither stimulus was able to enhance O_2^- as well as MDA production in the presence of iron-saturated DFO.

Effects of DFO on superoxide production by PMNs upon PMA stimulation

Compared to controls, preincubation of PMNs with DFO at study concentrations ranging from 0.125 mg/mL to 10 mg/mL significantly enhanced O_2^- production when PMA stimulation occurred (Table 1; Figures 1 and 2). Maximal intracellular O_2^- production was significantly achieved across the range of DFO concentrations from 0.125 mg/mL to 5 mg/mL, while maximal extracellular O_2^- production was observed across the range from 0.25 mg/mL to 0.75 mg/mL.

Table 1. Intracellular superoxide (NBT DK test), extracellular superoxide (cytochrome *c* reduction), and lipid peroxidation by PMNs preincubated with various of DFO concentrations upon PMA stimulation.

DFO mg/mL	*NBT DK test T% 5x10⁵ PMN*	*Cyt. c reduction nmoles 5x10⁵ PMN*	*MDA nmoles 5x10⁵ PMN*
control	17.1±4.72	5.14±1.23	0.085±0.019
0.125 mg/mL	2.48±1.73*	6.93±1.27°	0.143±0.019*
0.25 mg/mL	0.03±1.10*	8.44±1.06*	0.154±0.018*
0.50 mg/mL	2.39±1.72*	7.54±1.23*	0.143±0.023*
0.75 mg/mL	6.11±2.01*	7.25±0.98*	0.135±0.018*
1.00 mg/mL	8.18±2.76*	6.81±1.14°	0.125±0.019*
5.00 mg/mL	11.21±2.04*	5.77±1.10#	0.103±0.023#
10 mg/mL	12.95±3.17°	5.49±0.96#	0.100±0.018#

*Values are expressed as absolute values. Results are mean±SD of 10 separate experiments. *p<0.001 vs controls; ° p<0.01 vs controls; #p<0.05 vs controls.*

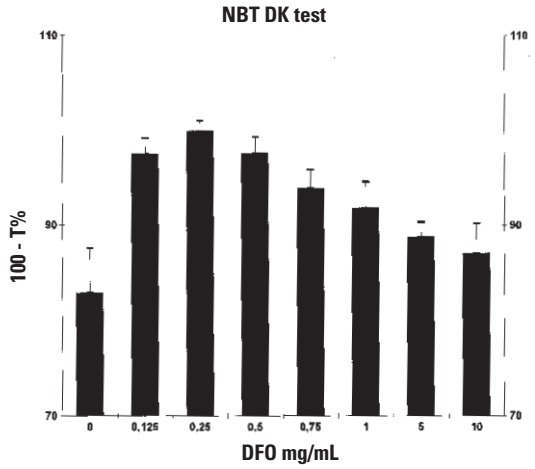

Figure 1. Intracellular O_2^- production (NBT DK TEST) by PMNs preincubated with various concentrations of DFO upon PMA stimulation. Values are expressed as a 100–T% for $5x10^5$ cells. Results are mean±SD of 10 separate experiments.

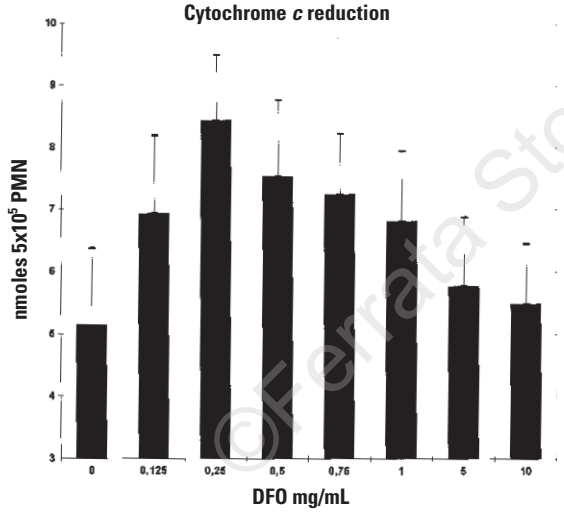

Figure 2. Extracellular O_2^- production (cytochrome *c* reduction) by PMNs preincubated with various concentrations of DFO upon PMA stimulation. Results are expressed as nmoles for $5x10^5$ cells. Results are mean ± SD of 10 separate experiments.

Effects of DFO on superoxide production by PMNs upon fMLP stimulation

In experiments dealing with fMLP stimulation, preincubation of PMNs with DFO at the above study concentrations did not alter baseline O_2^- values.

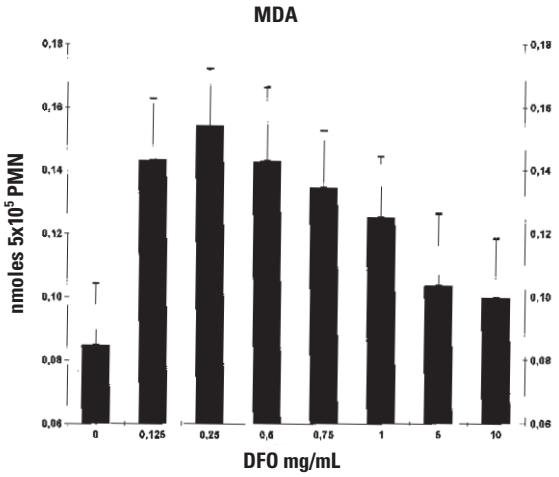

Figure 3. Lipid peroxidation (MDA assay) in PMNs preincubated with various concentrations of DFO upon PMA stimulation. Results are expressed as nmoles for $5x10^5$ cells. Results are mean ± SD of 10 separate experiments.

Effects of DFO on lipid peroxidation upon PMA stimulation

Compared to controls, preincubation of PMNs with DFO significantly increased MDA production when PMA stimulation occurred (Table 1, Figure 3). Maximal MDA production was achieved at DFO concentrations ranging from 0.125 mg/mL to 1 mg/mL.

Effects of DFO on lipid peroxidation upon fMLP stimulation

Preincubation of PMNs with DFO at any study concentrations had no effect on baseline MDA values.

Discussion

Since the early 1980s DFO has been suggested to play an antioxidant role under conditions in which the iron chelator scavenges hypochlorous acid or inhibits myeloperoxidase iodinating activity.[21-23] Nonetheless, trace metals such as iron do participate in the generation of hydroxyl radical formation through the reaction of O_2^- and H_2O_2 (Haber-Weiss reaction).[24,25]

In 1984, van Asbeck presented evidence for DFO-mediated enhancement of PMN function upon stimulation with *Staphylococcus aureus* and suggested that this could have been the result of protection against injury by an iron-dependent system that requires active metabolism.[16] On the basis of our experiments dealing with unsaturated and saturated DFO, neither iron-saturated nor unsaturated DFO alone was able to induce toxic oxygen species including O_2^-.

From this information, the response of PMNs to unsaturated DFO (without any physiologic source of artificial iron) was tested with two different stimuli to investigate the modulation of two different pathways in activating the respiratory burst in PMNs. The fact that prior exposure of PMNs to unsaturated DFO enhanced their superoxide anion release only upon stimulation with PMA reflects the differences between the cellular processes involved. At variance with PMA, fMLP did not show any change in PMN response, thus confirming that different metabolic pathways are individually modified during stimulation of the respiratory pathways.[26-29]

The respiratory burst consists of both cytoplasmic and membrane components and is deassembled in unstimulated cells. Some cytoplasmic components translocate to the cell membrane when triggered. Once this system is *switched on*, the membrane-associated NADPH oxidase system is activated. Thus it is not surprising that phosphorylation of a cytosolic protein (47 Kd) by protein kinase C plays a key role in this activation.[30] Unlike fMLP, PMA is known to activate protein kinase C directly.[31,32]

Whatever the mechanism(s) of unsaturated DFO-mediated enhancement of PMN functions, the *in vitro* effect was apparently dose-related. Indeed the oxidant's potential of DFO *in vivo* has been associated with certain concentrations of the drug when it is administered intravenously.[6-8]

Even though in the reported series of patients a cause-and-effect relationship between DFO and the pulmonary syndrome could not be proved directly, the temporal relationship between drug administration in high dosages and lung disease strongly supported such a relationship. Our *in vitro* findings may complement those encountered *in vivo*. On the basis of these data, such alternative chelation therapy should be monitored carefully with respect to pulmonary toxic effects, especially if attempts are made to escalate certain dosages.

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