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Original Contribution

FERROUS ION AUTOXIDATION AND ITS CHELATION IN IRON-LOADED HUMAN LIVER HepG2 CELLS

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Abstract—Ferrous ion (Fe^{2+}) is long thought to be the most likely active species, producing oxidants through interaction of Fe^{2+} with oxygen (O_2). Because current iron overload therapy uses only Fe^{3+} chelators, such as desferrioxamine (DFO), we have tested a hypothesis that addition of a Fe^{2+} chelator, 2,2′-dipyridyl (DP), may be more efficient and effective in preventing iron-induced oxidative damage in human liver HepG2 cells than DFO alone. Using ferrozine as an assay for iron measurement, levels of cellular iron in HepG2 cells treated with iron compounds correlated well with the extent of lipid peroxidation ($r = 0.99$ after log transformation). DP or DFO alone decreased levels of iron and lipid peroxidation in cells treated with iron. DFO + DP together had the most significant effect in preventing cells from lipid peroxidation but not as effective in decreasing overall iron levels in the cells. Using ESR spin trapping technique, we further tested factors that can affect oxidant-producing activity of Fe^{2+} with dissolved O_2 in a cell-free system. Oxidant formation enhanced with increasing Fe^{2+} concentrations and reached a maximum at 5 mM of Fe^{2+}. When the concentration of Fe^{2+} was increased to 50 mM, the oxidant-producing activity of Fe^{2+} sharply decreased to zero. The initial ratio of Fe^{3+}:Fe^{2+} did not affect the oxidant producing activity of Fe^{2+}. However, an acidic pH (< 3.5) significantly slowed down the rate of the reaction. Our results suggest that reaction of Fe^{2+} with O_2 is an important one for oxidant formation in biological system, and therefore, drugs capable of inhibiting redox activity of Fe^{2+} should be considered in combination with a Fe^{3+} chelator for iron overload chelation therapy.

Keywords—Iron autoxidation, Free radicals, Iron overload, Iron chelators

INTRODUCTION

Iron is essential for human life. Iron deficiency anemia is a common condition known to the medical profession for several centuries, though iron overload is mistakenly believed to be rare. Iron overload is an increase in total body iron generally exceeding 5 g. The normal levels of body iron range from 50–60 mg/kg in males, 35–40 mg/kg in females, and very low in children and young women [1]. Diseases that are related to or associated with iron overload can be exemplified by hereditary hemochromatosis, β-thalassemia, fibrosis, cirrhosis, and cancer [2–5].

Liver is the main organ for iron storage and metabolism. Iron-catalyzed oxidative stress is believed to be the main mechanism involved in the pathogenesis of organ dysfunction in iron overload [6]. In biological systems, it is often considered that ROS induced by iron originate from the interaction of iron with enzymatically and/or nonenzymatically generated superoxide ($O_2^{\bullet -}$) (Haber-Weiss reaction) and/or hydrogen peroxide (H_2O_2) (Fenton reaction). Measurements in liver cells have determined the steady state level of H_2O_2 to be approximately 10^{-8} M [7], and the steady state level of O_2 in vivo is about 10^{-5} M [8]. Assuming that the rate constant for oxidation of substrate by "$Fe^{2+} + O_2$" chemistry (Fe^{2+} autoxidation) is similar to Fenton reaction and that the oxidizable substrate concentration of a living system is about 1 M, it has been estimated that the rate of oxidation of oxidizable substrate by "$Fe^{2+} + O_2$" could be as much as 10^8 faster than the rate of oxidation by the Fenton reaction [9]. These results suggest that "$Fe^{2+} + O_2$" chemistry is probably the most important route for free radical biology of iron. In fact, $O_2^{\bullet -}$ and H_2O_2 may also

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be produced directly from dissolved oxygen (O_2) in aqueous media in the Fe^{2+}-mediated autoxidation reactions as follows:

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\bullet -} \quad (1)$$

$$Fe^{2+} + O_2^{\bullet -} + 2\,H^+ \rightarrow Fe^{3+} + H_2O_2 \quad (2)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet} \quad (3)$$

It is known that the activation of oxygen by iron is subject to both kinetic and thermodynamic restraints [10], and therefore, reactions (1)–(3) as described are oversimplified. For example, it has been suggested that oxidants other than the hydroxyl radical ($OH^{\bullet}$), such as ferryl or iron oxo, may also be generated [9,11]. On the other hand, it is noteworthy that conversion of H_2O_2 and O_2 into the more reactive $OH^{\bullet}$ radical requires the participation of Fe^{2+}. Desferrioxamine (DFO), the only clinically approved drug in the U.S. for iron chelation therapy, seems to be limited because it binds tightly only to Fe^{3+}. That is probably the reason only partial inhibition by DFO occurred in Fe^{2+}-induced degradation of 5-hydroperoxymethyl-2′-deoxyuridine [12].

The goals of the present study were (i) to investigate the preventive effects of 2,2′-dipyridyl (DP), a chelator which favors the binding of Fe^{2+}, in combination with a Fe^{3+} chelator, DFO, on cellular iron status and oxidative damage induced by iron overload; (ii) to investigate the reactivity of Fe^{2+} toward O_2 in cell-free biological media. Because DFO is a chelator which favors the binding of Fe^{3+} ions and the kinetic of its chelation is slow [13], DFO treatment may leave the more active Fe^{2+} freely producing oxidants [14]. If this is the case, the combination of DFO with DP may be more efficient in iron overload treatment than DFO alone. Because of the high physiological ratio of $[O_2]/[H_2O_2]$ ($\geq 10^3$), $Fe^{2+} + O_2$ reaction is probably the most important in generating detrimental free radicals in vitro and in vivo comparing to Fenton or Haber-Weiss reactions [9]. We investigated how factors such as ligands, Fe^{2+} concentrations, and pH may affect the oxidant-producing activity of Fe^{2+} towards O_2 in a relatively simple aerated aqueous media. We expect that clarification of the redox pattern of Fe^{2+} ions in aqueous media may give a better insight into intracellular iron metabolism, and thereby, into a number of pathologic mechanisms due to iron overload.

MATERIALS AND METHODS

Materials

Ferrous sulfate heptahydrate ($FeSO_4{}^{\bullet}7H_2O$), ferric sulfate pentahydrate, 2,2′-dipyridyl (DP), desferrioxamine (DFO), potassium phosphates, 2′-deoxyguanosine (dG), 5,5′-dimethyl-1-pyrroline N-oxide (DMPO), thiobarbituric acid (TBA), sodium formate, butanol, glacial acetic acid, sodium acetate, 1,10-phenanthroline, and ferrozine kit were obtained from Sigma Chemical Co. (St. Louis, MO, USA) with highest purity available. All solutions were prepared using Milli-Q water (18 $M\Omega cm^{-1}$).

Cell culture and treatment with iron sulfates

Human liver HepG2 cells were obtained from American Type Culture Collection (ATCC HB8065, Rockville, MD, USA). HepG2 cells were cultivated in a 75-cm^2 flask (Fisher Scientific, Fair Lawn, NJ, USA) in 10 ml of alpha-Minimum Essential Medium (α-MEM) completed with 10% fetal bovine serum, 1% antibiotics, 1% L-glutamine and grown in an atmosphere of 95% air, 5% CO_2 at 37°C. At approximately 60% confluence, cells were pretreated with 0.5 mM Fe^{2+} or Fe^{3+} sulfates for 24 h. After washing, cells were treated with 0.5 mM DFO or 1.5 mM DP for an additional 24 h. Cells were also pretreated with iron chelators, followed by iron treatment with the same concentrations of iron and its chelators as described above. In case of the combination of DFO and DP for cellular pre- or posttreatments, a mixture of 0.5 mM DFO and 0.05 mM DP (molar ratio 10:1) was used, because the proportion of Fe^{3+}:Fe^{2+} was approximately 50:1 in the tissue culture media, in which HepG2 cells were cultured. Experiments were performed in quadruplicate. Cells were then trypsinized and cell viability was evaluated using Trypan blue exclusion assay. Cells were counted after each treatment using a hemocytometer and finally stored at −80°C for the following experiments.

Iron measurements in HepG2 cells

Immediately prior to iron measurements, control and iron-treated HepG2 cells were suspended at 5×10^6 cells/ml in a buffer containing 10 mM Tris, pH 7.2, 150 mM NH_4Cl, and lysed on ice with occasional vortexing [15]. Cell debris was removed by centrifugation at $10{,}000 \times g$ for 15 min. The supernatants were recovered and aliquoted for cellular iron analyses, as well as protein determination. Protein concentrations were determined using Bradford reagents (Sigma). Cell precipitates (matrix) were used for lipid peroxidation assays.

The iron levels in the cell supernatants were determined using commercially available kits (Sigma). The principle of the test is that ferrozine, a sulfonated derivative of diphenyltriazine, forms a water-soluble magenta-colored complex with iron (II). At acidic pH and in the presence of suitable reducing agents, transferrin-bound

but not ferritin-bound iron dissociates to form Fe^{2+}. These Fe^{2+} ions, along with "free" or bioavailable iron, if present in cells, react with ferrozine to produce a magenta-colored complex with absorption maximum at 560 nm. The difference in color intensity at this wavelength, before and after the addition of ferrozine, is proportional to the concentration of iron in cells that is present in bioavailable iron and transferrin.

Measurements of lipid peroxidation

The levels of lipid peroxides in the iron-treated cells were measured by a thiobarbituric acid (TBA) reaction, as previously described [16]. Cell matrix samples were suspended in 0.1 ml PBS at 10^7 cells/ml and then mixed with 0.03 ml of 10% SDS and 0.6 ml of 0.4% TBA in 10% acetic acid, pH 5. The mixture was adjusted to 0.8 ml with distilled water, and then heated to 90°C for 1 h. After cooling, 0.8 ml of butanol was added and the mixtures were shaken vigorously for 20 s. After centrifugation at 1500 rpm for 15 min, the fluorescence intensities of TBA-reactive substances (TBARS) such as malonaldehyde and monoaldehydes in the butanol phase were measured at an excitation wavelength of 515 nm (bandwidth 4.5 nm), and an emission wavelength of 553 nm (bandwidth 9.0 nm) using a fluorescence-chemiluminescence microplate reader (Gemini, Molecular Device) [17]. The results of lipid peroxidation or iron levels are expressed as relative fluorescence units (RFU) or nmoles per mg proteins.

Oxidant-producing activity of $FeSO_4$ in various media

An experimental protocol designed to test for the presence and reactivity of ROS (e.g., $OH^{\bullet}$) induced by Fe^{2+}-autoxidation is illustrated in reactions (4) and (5).

$$OH^{\bullet} + HCO_2^- \rightarrow H_2O + CO_2^{-\bullet} \quad (4)$$

$$CO_2^{-\bullet} + DMPO \rightarrow DMPO\text{-}CO_2^{-\bullet} \quad (5)$$

In the presence of formate anions, ROS such as $OH^{\bullet}$, react with formate to produce carboxylate radicals ($CO_2^{-\bullet}$). If formed in solution, the carboxylate radicals, whose lifetime is in the order of 10^{-9} s, can be trapped by the spin trapping agent, DMPO. This creates a radical adduct (DMPO-$CO_2^{-\bullet}$), whose lifetime is at least 1 h, if there are no stronger reducing species present in the medium. The quantity of formed ROS is evaluated by the intensity of the ESR signal (DMPO-$CO_2^{-\bullet}$). The ESR signal intensities presented in this article are expressed in arbitrary units (a.u.). One thousand a.u. corresponds to 4.14×10^{18} radicals per liter, as previously reported [18].

Our experiments were carried out as follows: Sodium formate (4 M) was dissolved in potassium phosphate buffer (PB, 1 M, pH 7.4), Milli-Q water, or in other media. DMPO solution (100 mM) was prepared as previously described [19]. After mixing DMPO (1 ml) with formate (0.5 ml), 0.5 ml of freshly prepared $FeSO_4$ solution of various concentrations were immediately added to the mixture (total volume: 2 ml). The reaction proceeded at 37°C in the dark. Aliquots of the reaction were withdrawn at intervals of 5, 30, and 60 min after the addition of DMPO and filtered through a 0.65 μm filter (Cellulose acetate, Prolabo, Paris, France). Detection of radical adducts was performed by ESR spectroscopy (Varian CSE E-3 instrument). Control experiments utilized 0.5 ml of Milli-Q water instead of 0.5 ml of $FeSO_4$ solution.

The pH of the media in the presence of sodium formate (1 M) and different concentrations of $FeSO_4$ but without DMPO were also measured. The role of the Fe^{3+}:Fe^{2+} ratio on the reactivity of Fe^{2+} was studied by keeping the Fe^{2+} concentration constant (1 mM final) and varying Fe^{3+} concentration. $Fe_2(SO_4)_3$ was freshly dissolved in Milli-Q water and then mixed with the freshly prepared $FeSO_4$ in various proportions; 0.5 ml of the mixture was immediately added to the 0.5 ml formate and 1 ml DMPO. The intensities of DMPO-$CO_2^{-\bullet}$ signal were measured as described above.

Oxidation of 2′-deoxyguanosine (dG) by $FeSO_4$ and oxygen under various pHs

To study the effect of pH on the oxidant-producing activity of $FeSO_4$, 1 mM dG was incubated with 1 mM Fe^{2+} for 24 h at pH 1 (100 mM HCl), pH 3.0 (1 mM HCl), pH 4.5 (100 mM PB), pH 6.0 (100 mM PB), and pH 7.4 (100 mM PB). Blanks contained everything except Fe^{2+}. Oxidation of dG causes formation of 8-oxo-dG, which is detectable electrochemically. The reaction was stopped by adding Chelex-100 (BioRad, Hercules, CA, USA) to remove Fe^{2+} and then filtered through an ultrafree-MC filter (5000 NMWL, Millipore, Bedford, MA, USA) to remove Chelex-100 resin. Both dG and 8-oxo-dG passed through the filter, and the recovery of both nucleosides was >99%. The filtrates were analyzed by HPLC coupled with an electrochemical (EC) detector (Waters 4600), as previously reported [20]. In brief, the reaction mixture was separated on an ODS column with 6% methanol in 50 mM sodium acetate buffer (pH 5.1) at a flow rate of 1 ml/min. 8-oxo-dG was monitored by EC detector set at 600 mV and 0.2 nA, while dG was detected by UV at 254 nm. The peaks of 8-oxo-dG and dG were integrated by an integrator with two channels (Waters 746, data module). Since the starting concentration of dG was the same in all experiments, the amount

of 8-oxo-dG was expressed as arbitrary units (a.u.) after subtracting the blank values of 8-oxo-dG from the corresponding pH media.

Statistics

Experiments with HepG2 cells were done in quadruplicate. Results on levels of iron and lipid peroxidation from cells pretreated with chelators (e.g., DFO) followed by iron (e.g., Fe^{3+}) or from cells pretreated with iron (e.g., Fe^{3+}) followed by chelators (e.g., DFO) were similar and were combined for statistical analyses. Therefore, the average ± standard error (SE) in HepG2 cells were from at least eight experiments. Student's *t* test with two-tails and equal variances was used for statistical significance calculation. Results of ESR were an average of two experiments.

RESULTS

Distribution of Fe^{3+} and Fe^{2+} in tissue culture media

Ferrous sulfate ($FeSO_4$, 0.5 mM) or ferric sulfate [$Fe_2(SO_4)_3$, 0.5 mM] were added to the tissue culture media containing 10% FBS, in which HepG2 cells were cultured. After 5 h treatment, media were collected. Fe^{2+} was measured spectrophotometrically using DP-Fe^{2+} complex at 520 nm, and Fe^{3+} was measured using DFO-Fe^{3+} at 419 nm. We found that Fe^{2+} represented 1.8 ± 0.4% ($n = 3$) of total 0.5 mM Fe^{2+} added into the media in the form of $FeSO_4$, and 98.1 ± 0.3% of iron were in Fe^{3+} ions. When $Fe_2(SO_4)_3$ was used (1 mM Fe^{3+}), the distribution of iron was 1.4 ± 0.3% ($n = 3$) as Fe^{2+} and 98.6 ± 0.3% as Fe^{3+}, respectively. Because of the sensitivities of the available methods, we were unable to determine the concentrations of Fe^{2+} and Fe^{3+} ions within the cells. However, the media we used for the distribution study of iron oxidation states were from that in which the HepG2 cells were grown. These results strongly suggest that bioavailable iron in cells may exist in both Fe^{2+} and Fe^{3+} forms. Therefore, a Fe^{2+} chelator is probably needed to render Fe^{2+} inactive.

Correlation between lipid peroxidation and cellular iron levels

Figure 1 summarizes the measurements of levels of lipid peroxides and cellular iron in a total of 88 experiments that were performed. Human liver HepG2 cells were treated with various amounts of Fe^{2+} or Fe^{3+} sulfates under various conditions (e.g., in the absence or presence of DP and/or DFO chelators, different incubation time, addition of iron chelators simultaneously with iron or pre- or post-iron treatment). The supernatants were collected for cellular iron measurements using ferrozine as an assay. Levels of lipid peroxides in the precipitates (cellular matrix) were measured by thiobarbituric acid reactions. Figure 1 shows an almost perfect correlation between lipid peroxidation and cellular iron levels (correlation coefficient $r = 0.99$ after log transformation). These results provide strong evidence that iron is responsible for the oxidative damage in cells.

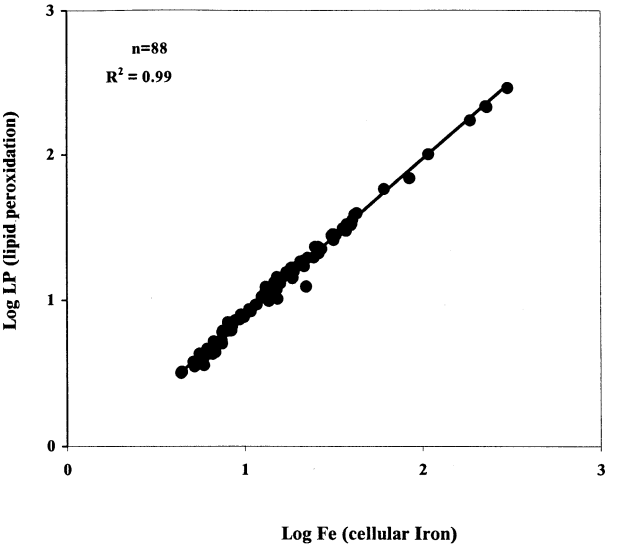

Fig. 1. Levels of lipid peroxidation in HepG2 cells are correlated with cellular iron levels after log transformation. Cellular iron was expressed as nmoles Fe/mg protein and lipid peroxidation as relative fluorescence units/mg protein.

Effects of iron chelators on cellular iron levels

Cellular iron levels were measured using the ferrozine assay. Figure 2 shows that all treatments with iron, either Fe^{2+} or Fe^{3+} with or without iron chelators, significantly enhanced iron levels in comparison with control HepG2 cells (data expressed as fold control), while chelators alone or together had no effects on cellular iron levels. Treatment of cells with Fe^{3+} + DFO or Fe^{2+} + DP (under conditions that cells pretreated with iron for 24 h, after washing, followed by the specific iron chelator for additional 24 h or pretreated with chelators followed by iron treatment) did decrease, to some extent, iron levels comparing to iron treatments alone, but those decreases were not statistically significant. A mixture of DFO + DP in a molar ratio of 10:1 was used because the distribution of Fe^{3+}:Fe^{2+} was approximately 50:1, as shown above. Incubation of cells with the same mixture (DFO + DP at 10:1) decreased iron level in Fe^{2+}-treated cells but increased that in Fe^{3+}-treated cells. Because ferrozine assay measures iron from transferrin and "free" or bioavailable iron, if present in cells, we tested and

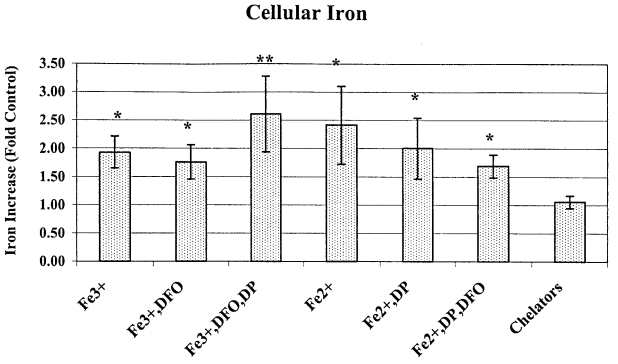

Fig. 2. Effects of DP, DFO, or DP + DFO on cellular iron status. Data were obtained from at least eight experiments and were presented as mean ± SE. Data in the bar marked "chelators" were the average of three sets of experiments: DP alone ($n = 4$), DFO alone ($n = 4$), and DFO + DP (molar ratio 10:1, $n = 4$). Levels of iron in cells treated with chelators were similar and were combined for data analysis. *Significantly different from the control ($p < .05$); **Significantly different from the control ($p < .01$); by Student's *t*-test.

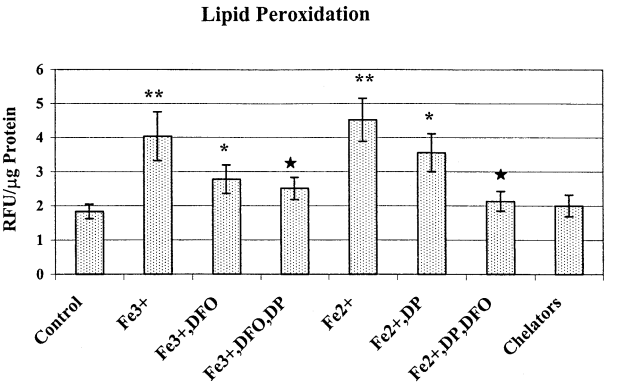

Fig. 3. Preventive effects of DP, DFO, or DP + DFO on lipid peroxidation. Data were obtained from at least eight experiments and were presented as mean ± SE. Levels of lipid peroxidation in cells treated with chelators (DFO, DP, or DFO + DP) were similar and were combined for data analysis. *Significantly different from the control ($p < .05$); **Significantly different from the control ($p < .01$); ★Significantly different from the iron-only treatment ($p < .05$); by Student's *t*-test.

found that a carefully prepared ferrioxamine (0.5 mM DFO-Fe^{3+} complex in 1:1 molar ratio) also released 89.6% iron, and DP-Fe^{2+} complex in molar ratio 3:1 and 1:1 (0.5 mM Fe^{2+}) released 45.3% and 75% iron, respectively. These results suggest that iron status in cells measured by ferrozine reflects the iron levels rather than "redox active" form of iron in HepG2 cells. To evaluate the protective role of DFO + DP from redox active iron-induced oxidative damage, levels of lipid peroxides in the same iron-treated HepG2 cells were measured.

Preventive effects of iron chelators on lipid peroxidation

Figure 3 shows that levels of TBARS were significantly higher in both Fe^{3+}- and Fe^{2+}-treated cells than those in control cells ($p < .01$ by Student's *t*-test). DFO or DP alone had no effect on lipid peroxidation. Pre- or posttreatment of the HpeG2 cells with either DFO or DP prevented iron-induced lipid peroxidation to some extent. It is notable that the treatment with DFO or DP showed a decrease in lipid peroxide levels when compared to the levels in only iron-treated cells, but these decreases were statistically insignificant. Although levels of lipid peroxides in groups treated with iron and one of the two chelators were still significantly higher than those in control cells ($p < .05$), the combination of DFO + DP had the most pronounced effects in preventing cells from iron-induced oxidative damage. The combination of these two chelators caused a significant decrease ($p < .05$) in levels of lipid peroxides when compared to the groups treated with iron only. These results prove our hypothesis that, in addition to DFO, a Fe^{2+} chelator may be needed for an iron overload chelation therapy.

Oxidant-producing activity of Fe^{2+} in Milli-Q water

In the presence of formate, ESR spectra exhibited an increased signal in the intensity of DMPO-$CO_2^{-\bullet}$ as a function of Fe^{2+} concentration (Fig. 4). The signal was increased with Fe^{2+} concentration up to 5 mM, then decreased at 10 mM, and disappeared at 50 mM (Fig. 4). The maximal intensities of the DMPO-$CO_2^{-\bullet}$ signal given by 5 mM $FeSO_4$ were 2,210 a.u. and 3,680 a.u. at 30 and 60 min withdrawals, respectively. The pHs of the reaction mixtures in the absence of DMPO were also

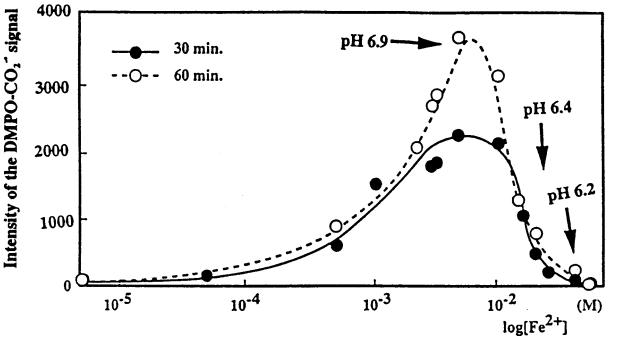

Fig. 4. Evolution of the intensity of DMPO-$CO_2^{-\bullet}$ signal (a.u.) in water as a function of Fe^{2+} concentration and pH of the media. The constants for DMPO-$CO_2^{-\bullet}$ were as follows: g = 2.0055, a_N = 15.6 G, a_H = 19.0 G. The ESR specifications were as follows: Varian CSE instrument, field set: 3380 G, scan range: 100 G, microwave power level: 10 mW, time constant: 1 s. 1000 a. u. corresponds to 4.14×10^{18} radicals/L.

measured. As shown in Fig. 4, the addition of $FeSO_4$ (5 mM) to sodium formate (1 M, pH 8.6 in water) caused a decrease in pH to 6.9. Under the same conditions, 20 and 50 mM of $FeSO_4$ gave pHs of 6.4 and 6.2, respectively.

In the absence of formate, no DMPO-$OH^{\bullet}$ signal was observed within the range of the concentrations of Fe^{2+} tested (50 μM to 50 mM). This may be due to a number of factors: (i) Oxidants other than $OH^{\bullet}$ may be formed. Ferryl, a high valent iron-oxygen complex ($Fe^{IV} = O$) should be considered in the redox reaction of Fe^{2+} with O_2; (ii) Reaction (1) of the Fe^{2+} autoxidation is a rate-limiting step. Without adding formate as a target molecule for ROS, the Fe^{2+} autoxidation may have occurred very slowly because of the acidic pH of $FeSO_4$ solution in water.

Increasing the spin trapping agent DMPO final concentration from 50 to 100 mM did not significantly intensify the signal of DMPO-$CO_2^{-\bullet}$ for 0.5 mM Fe^{2+} (increases from 560 and 800 a.u. to 600 and 975 a.u. at 30 and 60 min withdrawals, respectively). By decreasing the concentration of the target molecule formate from 1 M to 0.1 M, the DMPO-$CO_2^{-\bullet}$ signal intensity decreased to one third of the original. These results indicate that the chosen concentrations of formate (1 M) and DMPO (50 mM) are appropriate for our experiments. Interestingly, in the absence of O_2 or in the presence of *o*-phenanthroline (an Fe^{2+} chelator, molar ratio to Fe^{2+} 2:1; Fe^{2+} at 0.5 mM) or DFO (a Fe^{3+} chelator, molar ratio 1:1), no ESR signal was observed. These results indicate that (i) the oxidative activity arose from an oxidative species through O_2 interaction; and (ii) the formation of the oxidizing species can be inhibited by either a strong Fe^{2+} chelator or Fe^{3+} chelator.

Oxidant-producing activity of $FeSO_4$ in other media

In phosphate buffer (PB) and in the absence of formate, we did not observe the DMPO-$OH^{\bullet}$ signal at any of the Fe^{2+} concentrations tested. In contrast, two new signals appeared with intensity being related to the concentration of phosphate anions (Fig. 5). At 250 mM PB and 5 mM Fe^{2+}, we observed these signals (symbolized here as $P_1^{\bullet}$ and $P_2^{\bullet}$) with an intensity of around 100 a.u. in the aliquot withdrawn at 5 min. These signals decreased as a function of time shown by withdrawals at 30 and 60 min. At 50 mM PB, only $P_1^{\bullet}$ radical adduct with a weak intensity was observed.

In the presence of formate, similar patterns of Fe^{2+}-induced oxidant formation as in water were also observed in phosphate buffer as well as in α-MEM containing 20% serum. Table 1 summarizes the concentrations of Fe^{2+} giving the maximal oxidant producing activity as evaluated by the intensity of the DMPO-$CO_2^{-\bullet}$ signal. Increasing PB concentration increased the intensity of DMPO-$CO_2^{-\bullet}$ for a given concentration of Fe^{2+} and also shifted Fe^{2+} concentration that gave the maximal oxidant-producing activity to a higher end. In the presence of complete α-MEM medium (20% serum), the DMPO-$CO_2^{-\bullet}$ signal intensities were decreased for all tested Fe^{2+} concentrations in comparison with those obtained in water or in PB.

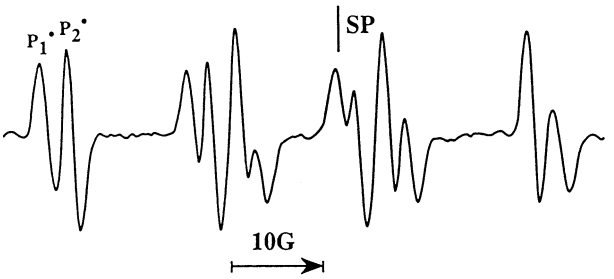

Fig. 5. Appearance of two new ESR signals due to the $P_1^{\bullet}$ and $P_2^{\bullet}$ free radical adducts trapped by DMPO in the presence of phosphate buffer (250 mM) and $FeSO_4$ (5 mM) without formate. SP: strong pitch as a reference compound, g = 2.0028.

Effects of the initial ratio of Fe^{3+}:Fe^{2+} and pH on oxidant-producing activity of $FeSO_4$

Because of the distribution of Fe^{3+} and Fe^{2+} in different media, we studied the effect of the initial Fe^{3+}:Fe^{2+} ratio on the oxidant-producing activity of $FeSO_4$. The concentration of Fe^{2+} was kept constant at 1 mM and the concentrations of Fe^{3+} were varied. We found that the initial Fe^{3+}:Fe^{2+} ratio exceeding 10:1 did inhibit the formation of DMPO-$CO_2^{-\bullet}$ in water, but not in PB (data not shown). Therefore, the ratio of Fe^{3+}:Fe^{2+} cannot alter the oxidant-producing activity of Fe^{2+} in biological systems, which are buffered.

Using 8-oxo-dG as an indicator of oxidant formation induced by Fe^{2+} autoxidation, Fig. 6 shows that Fe^{2+}

Table 1. Chemical Reactivity of Fe^{2+} Towards O_2 in Different Media[a]

		DMPO-$CO_2^{-\bullet}$ Intensity (a.u.)	
Media	Fe^{2+} (mM)[b]	30 min	60 min
H_2O	5	2210	3680
10 mM PB	5	4125	4200
50 mM PB	10	10,850	7400
250 mM PB	25	10,250	10,200
α-MEM with 20% serum	5	1190	1840

[a] Chemical reactivity of Fe^{2+} towards O_2 was tested in the media listed above. Similar patterns as shown in Fig. 4 were obtained for all media tested. Data presented here are an average of two experiments.
[b] Concentration of Fe^{2+} giving the maximal oxidant-producing activity.

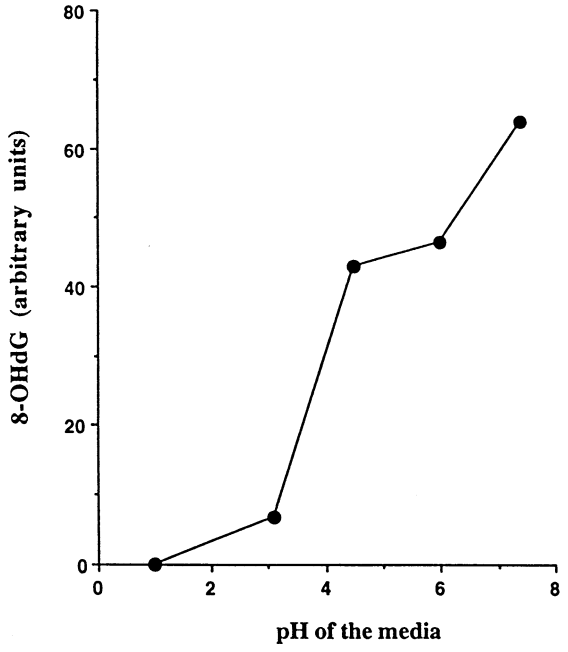

Fig. 6. ROS formation resulting from interaction of Fe^{2+} with O_2 as a function of pH. One mM dG was incubated with 1 mM Fe^{2+} for 24 h at pH 1 (100 mM HCl), pH 3.0 (1 mM HCl), pH 4.5 (100 mM PB), pH 6.0 (100 mM PB), and pH 7.4 (100 mM PB). The data presented were from an average of two experiments.

autoxidation resulting in ROS formation is very slow at pH below 3.0, but very fast at pH values above 3.

DISCUSSION

Iron overload is due to the amount of iron entering the body exceeding the amount lost over a long period of time. Phlebotomy is the therapy of choice in hereditary hemochromatosis. However, venesection is not an option for iron overload caused by a lifelong transfusion-dependent anemia such as β-thalassemia-major. Therefore, chelation therapy is the only way to remove excess iron [21,22]. DFO, which forms a hexadentate complex with Fe^{3+} in a molar ratio of 1:1, is the only clinically approved drug in the U.S. for iron chelation therapy. It has been known for a long time that the chelation of Fe^{3+} by DFO is kinetically slow and often takes long hours to completely complex Fe^{3+} ions in serum [13]. Moreover, DFO is poorly absorbed from the gut. Parenteral routes of administration are the only realistic methods of delivery. It has been shown that DFO-Fe^{3+} complex can have pro-oxidant effects, potentiating *tert*-butyl hydroperoxide-induced lipid peroxidation [23]. The high cost, parenteral method of administration, toxicity, and frequent patient noncompliance have led to a search for safe and orally active alternative. Other chelating agents, such as deferiprone (L1), an orally absorbed bidentate hydroxypyridinone Fe^{3+} chelator, are currently in clinical trials [24]. The combination use of DFO and L1 has been proposed with the highly permeant and fast chelating L1 acting as an intracellular chelator shuttle and the less permeant DFO serving as an extracellular iron sink [25].

Fe^{2+} is likely to be one of the active species in inducing oxidant formation, although our studies indicate that the amount of Fe^{2+} in biological media is relatively small compared to the amount of Fe^{3+} (1:50). Conversion of H_2O_2 and O_2 into the more reactive $OH^{\bullet}$ radical requires the participation of Fe^{2+}. Because of the dynamic exchange of Fe^{3+} and Fe^{2+}, it is possible that the presence of DFO or L1 gradually shifts the balance of free Fe^{2+} in biological media to Fe^{3+}-DFO or L1 complexes. However, DFO or L1 chelation therapy seems to be limited because it binds only to Fe^{3+}, and does not completely inhibit the oxidative damage mediated by Fe^{2+} [14].

DP and 1,10-phenanthroline are two Fe^{2+} chelators that were shown capable of inducing iron excretion, suggesting that a pool of Fe^{2+} may exist in cells [26]. We used DP because DP is less toxic (LD_{50} i.p. in mice: 200 mg/kg or 1.28 mmoles/kg) than 1,10-phenanthroline (LD_{50} i.p. in mice: 75 mg/kg or 0.42 mmoles/kg) [27]. Moreover, *1,10*-phenanthroline may potentiate the redox activity of copper [28,29]. In our in vitro studies, we did not observe significant effects of DFO and DP chelators in decreasing cellular iron levels in iron-treated cells (Fig. 2). This may be due to (i) high concentration of iron in the extracellular media; or (ii) the difficulty of cells in pumping out iron complexes through cell membrane. In vivo iron excretion by these chelators was shown to be effective [27]. Another reason we found is that ferrozine assay also measures part of iron released from DFO and DP-iron complexes under acidic assay conditions. Therefore, the iron levels measured by ferrozine reflect the total iron levels in the HepG2 cells, including iron released from transferrin, bioavailable iron if present, and iron released from DFO or DP-iron complexes. In the presence of high concentration of ascorbic acid (0.5 M), we found that iron can be released from ferritin and detected by the ferrozine assay (data not shown).

An increase in total iron levels in cells enhanced the risk of cells being oxidatively damaged, as shown by the correlation between cellular iron levels and lipid peroxidation (Fig. 1). DFO-Fe^{3+} and DP-Fe^{2+} complexes have been shown to possess some pro-oxidant properties [23], although they are not as potent as "free" iron in generating oxidant formation. Our results on lipid peroxidation indicate that a combination of DFO with DP, an Fe^{2+} chelator that tightly binds to Fe^{2+}, thus inhibiting redox activity of Fe^{2+}, much more efficiently prevents tissue damage in iron overloaded HepG2 cells than DFO alone.

These results validate our hypothesis that, in addition to an Fe^{3+} chelator, such as DFO, an Fe^{2+} chelator is needed for an effective iron chelation therapy, at least in terms of protection from iron-induced lipid peroxidation.

In the present study, we have shown that ROS formation as revealed by DMPO-$CO_2^{-\bullet}$ signal can originate from the interaction of Fe^{2+} with O_2. This finding is in a very good agreement with the observation of Qian and Buettner [9]. In the absence of O_2 or in the presence of *1,10*-phenanthroline or DFO, no ESR signal was observed, indicating that Fe^{2+} and Fe^{3+} and O_2 are essential elements for ROS formation. The high concentrations of iron used in our experimental system are relevant to the liver iron level (LIL) in iron overload patients, since LIL can be above 400 μmoles of iron per g of dry liver tissue [30]. Assuming that one gram of dry liver tissue is equivalent to 2.5 ml of cell volume of fresh liver tissue, the liver iron concentration within cells can be as high as 160 mM.

The data shown here (e.g., Fig. 4) should be viewed simply as an observation of the reactivity pattern of Fe^{2+} towards O_2 without elaboration needed for a mechanistic study. Nonetheless, it showed complexity of the Fe^{2+} reaction in a simple mixture, a system much simpler than the biological system, such as liver. Our simplest reaction system contained $FeSO_4$ and O_2 as reactants that are readily bioavailable in biological systems, sodium formate as a target molecule, and DMPO as a spin trapping agent. However, many factors, such as coordination of iron with formate, formation of oxyhydroxides, or degradation of DMPO radical adducts, can affect the reactivity of Fe^{2+} towards O_2 and cause the decrease in the ESR signal intensities at higher concentrations of Fe^{2+} (Fig. 4). One factor that we tried to study was the consumption of O_2. The initial concentration of O_2 was 0.25 mM, the concentration of O_2 solubilized in aqueous medium (3% v/v). Assuming that O_2 is totally consumed by higher Fe^{2+} concentrations, a plateau of oxidant-producing activity should be observed as a function of increasing Fe^{2+} concentration. In contrast, a sharp decrease in the DMPO-$CO_2^{-\bullet}$ signal was observed for those higher Fe^{2+} concentrations in all of the tested media (Fig. 4). Bubbling air or O_2 in the reaction mixture did not reverse the decrease in the ESR signal intensities (data not shown). These results indicate that the disappearance of DMPO-$CO_2^{-\bullet}$ was not caused by the total consumption of O_2 present in the media. In fact, it has been shown that the maximal oxygen uptake during autoxidation of various Fe^{2+} complexes is around 75% [31,32]. Others have estimated that the maximal O_2 consumption by 0.1 mM Fe^{2+}-complexes (e.g., EDTA, citrate) is 0.025 mM or 10% of the oxygen present in the solution [33,34]. Although acidic pH below 3.5 was shown to slow down the reaction of Fe^{2+} with O_2 (Fig. 6), the pH of the reaction mixtures at high Fe^{2+} concentrations were well above 3.5, in the range of 6.1–6.9, which favor the Fe^{2+} autoxidation [35]. Therefore, one possible explanation is that the oxidants produced by the interaction of Fe^{2+} and O_2 may be quenched by Fe^{2+} itself at the high concentrations as follows:

$$Fe^{2+} + OH^{\bullet} \rightarrow Fe^{3+} + OH^{-} \quad (6)$$

According to the reactions (1)–(3) and (6), a self-quenching reaction can be written as follows:

$$4\ Fe^{2+} + O_2 + 2\ H^{+} \rightarrow 4\ Fe^{3+} + 2\ OH^{-} \quad (7)$$

A total stoichiometry 4 Fe^{2+}: 1 O_2 can be proposed based on the reaction (7), though this stoichiometry is greatly dependent upon the nature of the iron chelator used, and can differ markedly from the 4:1 [33].

In conclusion, our studies indicate that ferrous ion in cells is one of the active species responsible for oxidant formation, leading to increased levels of lipid peroxidation. Fe^{3+} chelator DFO or Fe^{2+} chelator DP has protective effects on lipid peroxidation induced by iron treatments. However, it is the combination of both chelators that exerts the most significant effects in decreasing iron-induced lipid peroxidation. Our studies on the chemical reactivity of Fe^{2+} in the cell-free system clearly show that "$Fe^{2+} + O_2$" reaction is very active in forming oxidants. These results further strengthen our hypothesis that the combination of DFO or L1 with a strong Fe^{2+} chelator, such as DP, may be more efficient and effective in preventing tissue damage in iron overload patients.

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ABBREVIATIONS

8-oxo-dG—8-oxo-2′-deoxyguanosine
α-MEM—alpha-Minimum Essential Medium
a.u.—Arbitrary units
DFO—Desferrioxamine
dG—2′-deoxyguanosine
DMPO—5,5′-dimethyl-1-pyrroline N-oxide
FBS—Fetal bovine serum
L1—Deferiprone
LIL—Liver iron level
PB—Phosphate buffer
RFU—Relative fluorescence units
ROS—Reactive oxygen species
TBA—Thiobarbituric acid
TBARS—TBA reactive substances