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EVALUATION OF IRON BINDING AND PEROXIDE-MEDIATED TOXICITY IN RAT HEPATOCYTES

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Abstract—A novel assay was developed to determine subnanomolar amounts of Fenton-reactive iron (FRI) in biological tissues. FRI represents that pool of iron that is redox active and capable of participating in a model Fenton reaction. The FRI was used to identify a kinetically-defined cellular iron binding site. This site displays positive cooperativity, with apparent kinetic constants of K_d = 10.6 μM, B_{max} = 20.7 nmol/mg protein, and the Hill coefficient = 1.4. After addition of exogenous ferrous ammonium sulfate to hepatocytes, binding occurred within a few seconds and was stable for at least an hour. Free extracellular iron, but not bound iron, stimulated lipid peroxidation in hepatocytes. In contrast, bound but not free iron produced a concentration-dependent increase in *tert*-butyl hydroperoxide (TBH)-mediated toxicity, suggesting the toxicological relevance of bound, rather than free iron. Furthermore, the hydroxyl radical scavengers mannitol and 2-deoxyribose inhibited Fe^{2}/TBH-mediated lipid peroxidation, but not cell killing, suggesting that hydroxyl radical may not be involved in the critical toxic event. The divalent cations Mn^{2+} and Co^{2+} inhibited iron-mediated hepatocyte killing in the presence of TBH, but only if added prior to Fe^{2+}. Mn^{2+}, but not Co^{2+}, inhibited Fe^{2+}-mediated lipid peroxidation regardless of the order of addition. These results indicate the existence of a specific, kinetically-defined cellular iron binding site. Such binding is involved in peroxide-mediated toxicity, but independent of lipid peroxidation. The specific nature of this site and involvement with other forms of chemical intoxication or cellular iron homeostasis are unknown.

The critical role for iron in the production of reactive oxygen species and cellular oxidant stress has been the subject of several excellent reviews [1–4]. Intracellular iron, mostly ferric ion immobilized in a ferritin core, results from the transport of extracellular ferric proteins such as transferrin via receptor-mediated processes [5–10]. Since hydrolysis reactions with free ferric ion prohibit free concentrations in excess of about 10^{-17} M [11], the mobilization of protein-bound iron usually involves reduction to the ferrous state by a wide variety of reducing agents including $FMNH_2$, ascorbate, xenobiotics and free radicals [12–16]. Ferric ion may also be removed from ferritin without reduction by certain synthetic chelators such as 3-hydroxypyridinones [17].

Although ferrous ion concentrations may reach micromolar levels, especially in a reducing medium, it is doubtful that free unliganded iron represents a meaningful pool of iron in biological systems. Chelation sites in the form of sugars and amino acids may be present as low molecular weight species or in macromolecules. The potential for site-specific damage occurs if the redox potential and nature of the iron chelate allows for redox cycling. At present there are no appropriate methods to evaluate the chemical nature of distribution of redox-active ion binding molecules in the cell. However, it is possible to quantitate cellular redox-active iron, and to determine its toxicological relevance. This paper discusses a methodology for determining subnanomole amounts of Fenton-reactive iron (FRI§), and utilizes this assay to evaluate the kinetics of iron binding to hepatocytes and resulting toxicological consequences.

MATERIALS AND METHODS

Materials. Ascorbic acid, *tert*-butyl hydroperoxide (TBH), chelating resin (sodium form, 50–100 mesh), 2-deoxyribose, diethylenetriamine pentaacetic acid (DTPA), ferrous ammonium sulfate, hydrogen peroxide, mannitol and 2-thiobarbituric acid (TBA) were obtained from the Sigma Chemical Co. Chelating resin was treated by washing twice with 1 N HCl, followed by repeated washing with distilled water until the pH of the washing was 4–5.

Fenton-reactive iron. All reactions were performed using new plastic tubes, due to cation contamination and binding properties of glass. Stock solutions of 0.1 M potassium phosphate buffer (pH 7.0), 80 mM 2-deoxyribose, 1.0 M $MgCl_2$, 10 mM ascorbic acid (fresh daily), 100 mM H_2O_2, 4 M NaCl containing 20 μM EDTA and 2 mM HCl (4 M salt mix), and glass-distilled water were shaken with 1 g acid-washed chelating resin/100 mL solution, and stored in the presence of the settled resin. The reaction mixture consisted of 25 mM potassium phosphate

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§ Abbreviations: DTPA, diethylenetriamine pentaacetic acid; FRI, Fenton-reactive iron; TBARS, 2-thiobarbituric acid reacting substances; and TBH, *tert*-butyl hydroperoxide.

buffer, 20 μM ascorbic acid, 2.5 mM $MgCl_2$, 0.5 mM H_2O_2 and 0.2 M NaCl salt mix, in a final volume of 1.0 mL. The reaction was incubated at 20° with uniform room lighting, started by the addition of 16 mM deoxyribose, and stopped after 120 min by the addition of 10 mM DTPA. Blank sample values were obtained by adding 10 mM DTPA before 2-deoxyribose. The concentrations of ascorbate, EDTA, H_2O_2, and 2-deoxyribose were optimized, while $MgCl_2$ inhibited the reaction in a concentration-dependent manner. Magnesium was included in order to avoid complications that could arise by the unavoidable introduction of some magnesium in biological samples. Standards contained 0 to 1.4 nmol ferrous ammonium sulfate. After adding 0.1 mL of 50% trichloroacetic acid and 0.5 mL of 1% TBA in 0.28% NaOH, standards and samples (described below) were heated together in a dry block heater at 70° for 20 min. Tubes were centrifuged and 2-thiobarbituric acid reacting substances (TBARS) were estimated from the absorbance at 532 nm. A standard curve was performed for each experimental assay.

Free (extracellular) FRI in hepatocytes was determined by centrifuging cells at 10,000 *g*, and mixing 0.5 mL of supernatant solution with 0.5 mL of 2 M NaCl salt mix. The sample was heated at 70° for 30 min and stored at 4°. To determine FRI associated with hepatocytes (bound), cells (2 mL) were added to 0.25 g chelating resin and shaken, and the resin was allowed to settle (about 5 sec). A 1-mL aliquot of cells (contained in the supernatant suspension, since cells settled in 30–60 sec) was added to 1 mL 2 M NaCl salt mix heated at 70° for 30 min, and stored at 4°. When treated in this manner, samples were stable for at least 1 week. Before assaying for FRI, samples were sonicated and an aliquot was taken for protein determination. For the FRI assay, a 0.2-mL sample in 1 M salt mix was diluted in the reaction mixture to a final salt concentration of 0.2 M.

The kinetic and graphic evaluations of the binding data were performed using the commercially available software, EZ-FIT™ (Perrella Scientific, Inc., Springfield, PA). Protein was determined using the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL) according to the technical procedure supplied by the manufacturer.

Hepatocytes. Male Wistar rats (*ca.* 225 g) were allowed food and water *ad lib.* Hepatocytes were prepared by collagenase (grade II, Boehringer Mannheim, F.R.G.) digestion as described [18], followed by three times centrifugation (50 *g* for 2 min) and resuspension in fresh Krebs buffer. Cells were finally suspended at 10^6 cells/mL (about 1.2 mg protein/10^6 cells) in Krebs buffer containing 0.3% (w/v) HEPES and 1% (w/v) bovine serum albumin (pH 7.4) and incubated in rotating round bottom flasks at 37° under a carbogen atmosphere. Cell viability was assessed as previously described [19], either by trypan blue exclusion or by the release of lactate dehydrogenase activity [20] to the incubation medium. Initial viability was 85–90%.

For lipid peroxidation, at specified times after the addition of TBH, 1-mL aliquots of hepatocytes were treated with 0.1 mL of 50% trichloroacetic acid,

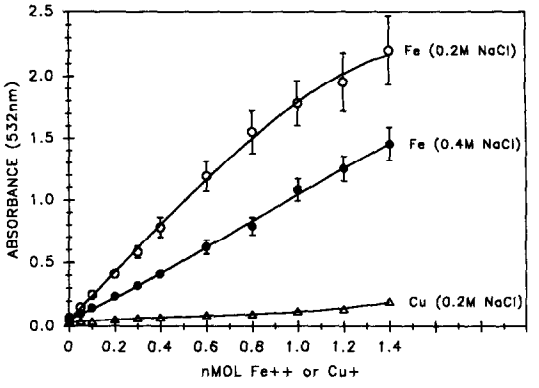

Fig. 1. Standard curves for the Fenton-reactive iron assay. Ferrous ammonium sulfate or cuprous chloride was added at the indicated concentrations to the reaction mixture described in Materials and Methods. After starting the reaction by the addition of 2-deoxyribose, it was important to maintain constant and uniform temperature and lighting conditions. The rate of the reaction increased precipitously at salt concentrations below 0.1 M, and with increasing temperature. The results are expressed as mean values ±SD for four standard curves. The absence of error bars indicates that the error lies within the symbol.

10 μL of 1 M butylated hydroxytoluene in DMSO (to prevent oxidation that may occur in the heating stage of the assay) and 0.5 mL of 1% TBA in 0.28% NaOH, and heated at 100° for 10 min. Samples were centrifuged for 10 min at 2500 *g*, and the supernatant solution was monitored at 532 nm for TBARs.

The concentration of TBH in the incubation medium was assayed at room temperature in a reaction mixture consisting of 50 μL sample and 0.5 mL of 1 mM ferrous ammonium sulfate. After 5 min, 1 mL of 2% KSCN in 0.1 N HCl was added and the mixed solution was incubated for 15 min. Absorbance at 490 nm was determined against a TBH standard curve, which was linear in the range of 0–40 nmol.

RESULTS

Figure 1 depicts standard curves for the FRI assay. At 0.2 M salt, the limit of detection was about 50 pmol iron and the reaction was linear to 0.8 nmol. At 0.4 M salt, the limit of detection was about 100 pmol and linearity was extended to 1.4 nmol. Higher salt concentrations decreased the sensitivity of the assay further, while extending the linear range. The reaction was quite sensitive to temperature, with the slope of the standard curve increasing at higher temperatures. It was also important to maintain uniform lighting conditions during the incubation. Copper was poorly reactive in this assay system (Fig. 1), while manganese and cobalt were non-reactive (data not shown). Hemoglobin and myoglobin did not react in this assay system at concentrations up to 0.5 mg/mL (data not shown).

The FRI assay was used to evaluate iron binding and reactivity in hepatocytes. Addition of sequential increments of ferrous ammonium sulfate to hepa-

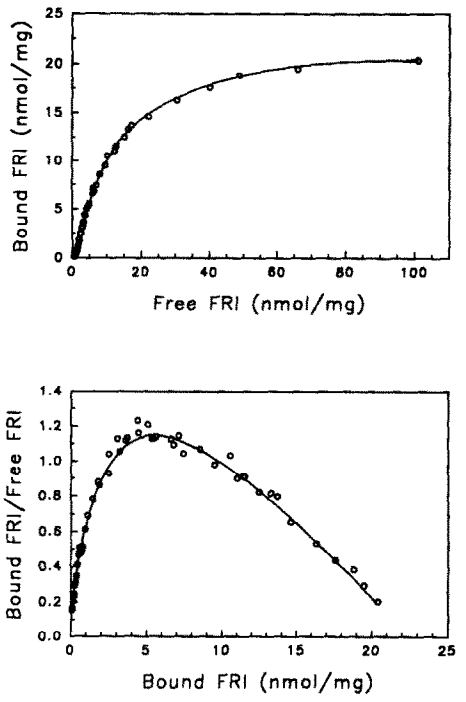

Fig. 2. Fenton-reactive iron binding to hepatocytes. Top panel: FRI bound to hepatocytes is plotted against free FRI (10,000 *g* supernatant activity). Bottom panel: A Scatchard plot of the same data. For both panels, a composite of the data is shown for six different experiments covering different concentration ranges of free FRI.

tocytes produced relative increases in FRI associated with cells (Fig. 2, top panel). Binding occurred within a few seconds, and the partition between FRI associated with cells and free in the medium did not change over the course of an hour (data not shown). The background value of FRI associated with hepatocytes was 1.6 ± 0.3 nmol FRI/mg protein. This may represent residual iron bound to hepatocytes, but since Chelex resin and DTPA washing did not decrease this value, it is more likely to be non-specific TBARS formed from cellular substrates rather than from 2-deoxyribose. This value was subtracted from FRI values obtained in the presence of iron before performing the Scatchard analysis. A Scatchard plot of the data (Fig. 2, bottom panel) was evaluated using the Hill equation, $b = B_{max}/[1 + (K_d/L)^N]$, where b = ligand bound, B_{max} = maximal ligand bound, L = ligand concentration, and N = the Hill coefficient. The plot revealed a positively cooperating binding site(s), with apparent kinetic constants of $K_d = 10.6 \pm 0.3$ μM FRI, $B_{max} = 20.7 \pm 0.3$ nmol FRI/mg protein, and a Hill coefficient = 1.39 ± 0.03.

Table 1 shows that increasing concentrations of iron in the presence of TBH enhanced TBH toxicity, and that this correlated with increased binding of FRI to hepatocytes. Washing the hepatocytes reduced free FRI in the medium, but neither reduced the amount of bound FRI nor diminished iron/TBH-mediated cell killing. In the absence of TBH, iron (up to 500 μM) had no effect on hepatocyte viability (data not shown). Table 2 shows that 25 μM iron did not affect the rate of disappearance of TBH from the incubation medium. Furthermore, iron in the presence of TBH stimulated the production of TBARS, which was associated with free iron in the medium. Washing cells to remove most of the free iron decreased TBARS to levels seen in the absence of added iron, but had no effect on iron-mediated cell killing which was associated with bound iron.

To determine whether hydroxyl radical may be involved in lipid peroxidation and cell killing, the hydroxyl radical scavengers mannitol and 2-deoxyribose were added to the hepatocyte incubation medium, followed by 25 μM ferrous ammonium sulfate and 0.5 mM TBH. These scavengers inhibited lipid peroxidation in a concentration-dependent fashion but had no effect on cell killing (Fig. 3), suggesting that hydroxyl radical was not mediating toxicity in this system.

The lack of a relationship between TBH/Fe^{2+}-mediated lipid peroxidation and cell killing was also observed in the presence of $MnCl_2$ and $CoCl_2$. Mn^{2+} inhibited lipid peroxidation whether added before or after the addition of Fe^{2+}, whereas Co^{2+} did not inhibit lipid peroxidation whether added before or after Fe^{2+} (Figs. 4 and 5, top panels). However, both Mn^{2+} and Co^{2+} inhibited cell killing in a concentration-dependent fashion, if added before Fe^{2+}. When added after Fe^{2+}, neither Mn^{2+} nor Co^{2+} affected TBH/Fe^{2+}-mediated cell death (Figs. 4 and 5, bottom panels).

DISCUSSION

The Fenton-reactive iron assay described is similar in principle to that reported by Gutteridge and Halliwell [21]. However, rather than measuring DNA damage and release of oxidized deoxyribose via an iron-bleomycin intermediate, the present assay utilizes 2-deoxyribose directly as the oxidizable substrate. The present system is about 50-fold more sensitive and is simpler for routine analyses. The mechanism of oxidation of 2-deoxyribose to TBARS by Fenton systems has been studied recently [22]. It was concluded that the system is complex and the ultimate oxidizing species may depend on the experimental conditions.

The catalytic roles for transition metals such as iron in producing oxidative stress through generation of reactive oxygen species is well established. However, there is some controversy regarding the nature of the species that may be responsible for mediating various toxicologic, events, such as lipid peroxidation, depletion of cellular antioxidants, DNA strand cleavage, enzyme or ion transport inhibition, or cell death. In the presence of peroxides or hydroperoxides, ferrous ion can produce hydroxyl and alkoxyl radicals through Fenton chemistry. In addition, ferrous ion can utilize molecular oxygen to generate the perferryl radical or other iron–oxygen complexes that can act as strong oxidants. The amount and the type of reactive oxygen species that are produced in any given system will depend on the availability, cellular location and nature

Table 1. Distribution of Fenton-reactive iron and *tert*-butyl hydroperoxide mediated cell killing in the presence of various iron concentrations

Iron added (μM)	Time to 50% death (min)	Bound FRI (nmol/mg protein)	Free FRI (μM)
A. In unwashed hepatocytes			
0	65	1.4	0.7
10	54	6.4	3.6
25	43	11.9	9.9
50	37	18.8	23.5
100	32	24.2	64.2
200	24	29.1	149
B. In washed hepatocytes			
0	56	1.5	0.1
10	50	7.2	0.1
25	41	14.4	0.3
50	33	20.6	0.7
100	26	26.4	1.4
200	21	32.1	4.4

Bound and free Fenton-reactive iron (FRI) were determined as described in Materials and Methods, after addition of the indicated concentrations of ferrous ammonium sulfate to hepatocytes at 1×10^6 cells/mL (about 1.2 mg protein/mL). Iron-dependent toxicity was determined by trypan blue exclusion in the presence of 0.5 mM TBH. Washed hepatocytes were prepared after a 1-min exposure to iron by centrifuging (4° for 5 min at 50 *g*) and resuspending in fresh Krebs buffer. Results are representative of three separate experiments.

Table 2. Effects of iron and hepatocyte washing on various parameters of *tert*-butyl hyderoperoxide mediated toxicity

Iron added (μM)	Wash	TBH $T_{1/2}$ (min)	TBARS ($A_{532\ nm}$)	Viability (%)
0	No	23	0.24	79
0	Yes	27	0.23	77
25	No	26	0.39	66
25	Yes	25	0.23	63

After the addition of 0.5 mM TBH, cells were sampled and centrifuged at 5-min intervals, and TBH was assayed in the supernatant solution. The log of the percent of TBH remaining was plotted against time, and the slope of this linear transformation was used to estimate the $T_{1/2}$ for the disappearance of TBH from the medium. Thiobarbituric acid reacting substances (TBARS) and viability were determined at 30 min after addition of TBH ± iron. Iron was added as ferrous ammonium sulfate. The results are representative of three separate experiments.

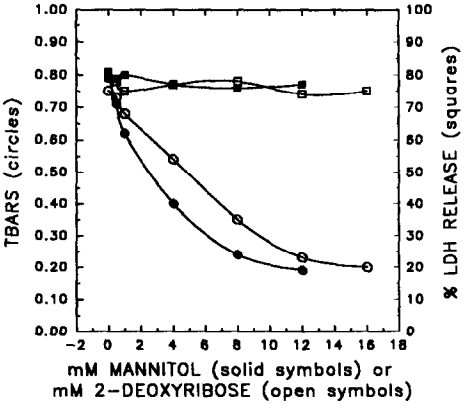

Fig. 3. Effect of hydroxyl radical scavengers on lipid peroxidation and cell killing by Fe^{2+}/TBH. Mannitol or 2-deoxyribose was added at the indicated concentrations prior to the addition of 25 μM ferrous ammonium sulfate and 0.5 mM TBH. After 60 min, cells were evaluated for lipid peroxidation (TBARS) and cell viability (LDH release) as described in Materials and Methods. The results are representative of two separate experiments.

(redox potential) of iron chelation sites, since the estimated concentrations of free ferrous and especially ferric ion are exceedingly low, in the range of 10^{-7} and 10^{-17} M, respectively [11]. When bound to a suitable biological ligand, iron ions may undergo cyclic reduction and oxidation, thus serving as site-specific centers for radical generation. It is clear that ferrous ion alone is insufficient to produce hepatocyte toxicity, since ≤500 μM had no effect in our system. Therefore, a Fenton reation involving iron and endogenous lipid hydroperoxides is either not occurring or is not responsible for cytotoxicity. However, in the presence of iron, TBH produced concentration-dependent lipid peroxidation and toxicity. The former, but not the latter, was inhibited by hydroxyl radical scavengers, suggesting that hydroxyl radical could be mediating lipid peroxidation, and that neither lipid peroxidation nor hydroxyl radical were directly involved in cytotoxicity. These conclusions support the findings of Rush *et al.* [23] with respect to the lack of a role of lipid peroxidation in the mechanism of acute cytotoxicity of isolated rat hepatocytes.

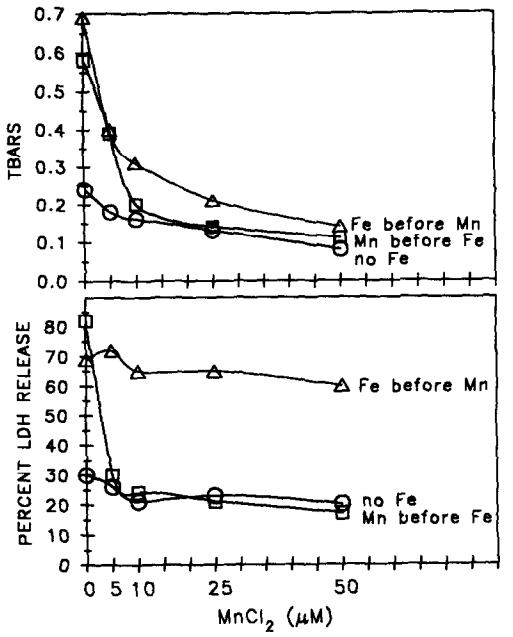

Fig. 4. Effect of manganese and iron on TBH toxicity. $MnCl_2$ was added at the indicated concentration either before or after the addition of 25 μM ferrous ammonium sulfate, followed by 0.5 mM TBH. Lipid peroxidation (TBARS) and cell viability (LDH release) were assayed at 60 min after TBH addition. The results shown are representative of two independent experiments.

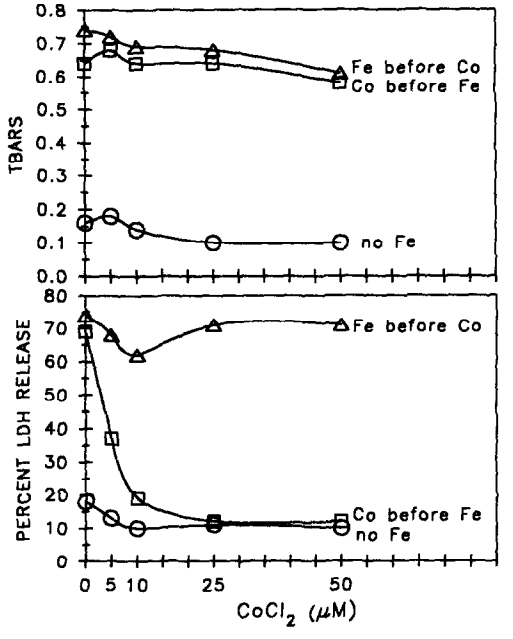

Fig. 5. Effect of cobalt and iron on TBH toxicity. $CoCl_2$ was added at the indicated concentration either before or after the addition of 25 μM ferrous ammonium sulfate, followed by 0.5 mM TBH. Lipid peroxidation (TBARS) and cell viability (LDH release) were assayed 60 min after TBH addition. The results shown are representative of two independent experiments.

The use of scavengers to clearly identify hydroxyl radical as the intoxicating reactive oxygen species has been questioned recently by Minotti and Aust [2]. It was demonstrated using purified phospholipid vesicles that Fe^{2+}/H_2O_2-initiated lipid peroxidation is stimulated by a hydroxyl radical scavenger such as benzoate that facilitates the oxidation of ferrous to ferric ions, while scavengers such as mannitol that minimize ferrous to ferric conversion inhibit lipid peroxidation [24]. The authors suggest that lipid peroxidation is mediated by a ferrous–dioxygen–ferric complex and discuss the evidence for and against the existence and toxicological implications of such a complex [2]. Therefore, the finding in the present study that lipid peroxidation but not cell toxicity was inhibited by mannitol or 2-deoxyribose may be explained by either (a) the involvement of alkoxyl or hydroxyl radical in lipid peroxidation but not cell toxicity, or (b) the ability of these scavengers to alter the redox state or decrease the rate of redox cycling of free, but not cellular bound iron that is responsible for toxicity but not lipid peroxidation.

Similarly, the inhibition of cell killing but not lipid peroxidation by Co^{2+} suggests that cell-associated iron is involved in cytotoxicity in the presence of TBH. The nature of this putative iron binding site is not clear. Most of the iron transported by plasma is bound to transferrin, and the iron–transferrin complex is internalized by hepatocytes by receptor-mediated and also fluid phase endocytosis [25, 26]. However, non-transferrin-bound iron which comprises less than 1% of the total plasma iron is taken up more rapidly than transferrin-bound iron [27] and appears to involve a facilitated diffusion process that is inhibited by cobalt and manganese [28]. Although this transporter may be associated with the iron binding site in our system, the evidence is somewhat against this possibility. First, hepatocyte-associated iron did not increase with time, as would be expected with binding to an iron-transport protein. Second, Co^{2+} strongly inhibited iron-mediated toxicity in our system, whereas Co^{2+} was a weak inhibitor of the passive uptake of iron [28]. However, it should be noted that our system would probably not detect the incorporation of limiting amounts of iron into non-Fenton-reactive ferritin by hepatocytes.

Another question involves the nature of reactive iron. The Scatchard plots for iron binding revealed positive cooperativity for a single type of binding site at low iron concentrations. That is, binding of one iron ion facilitated the binding of a second iron. One interpretation of such binding kinetics is that more than one (presumably two, since the Hill coefficient was 1.4) iron ion are involved in a reactive complex at the iron binding site. Such a species could be similar to the putative ferrous–dioxygen–ferric complex discussed previously. We propose a model to explain the results of the present study (Fig. 6). In this model, Fe^{2+} binds to a site that may also bind cobalt or manganese. After (or possibly before) binding, Fe^{2+} reacts with O_2 to form a perferryl complex (Fe^{2+}-$O_2 \leftrightarrow Fe^{3+}$-$O_2^-$) that may bind another Fe^{2+}, in a process that may be inhibited by cobalt or manganese. This membrane-bound iron–oxygen–iron complex may redox cycle in the

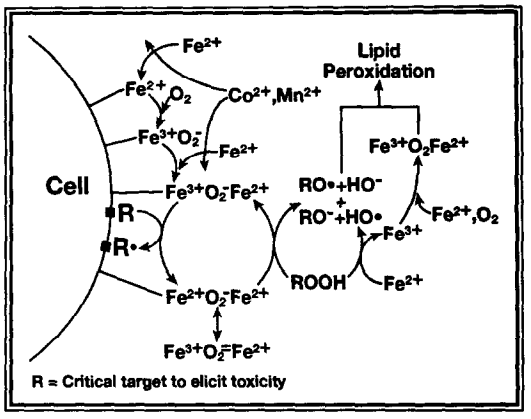

Fig. 6. Schematic representation of the possible nature of iron binding and redox-cycling in the course of hydroperoxide-mediated toxicity.

presence of a suitable oxidizing agent such as TBH, to produce radicals at the site of binding. Such a model would explain the positive cooperativity for iron binding as well as the lack of ferrous iron toxicity in the absence of an oxidant like TBH. The right-hand portion of the figure indicates that hydroperoxides and iron may generate oxidizing species that may peroxidize cellular lipids independently of the cellular iron binding site(s). Since iron binding is indicated to be a saturable process, while the generation of pro-lipid peroxidation radical species in bulk solution is not, the model suggests that lipid peroxidation may increase in toxicological relevance at elevated iron and hydroperoxide concentrations.

The model proposed in Fig. 6 does not attempt to identify the nature of hydroperoxide-mediated lipid peroxidation. Our results (Table 2) suggest that there are components of lipid peroxidation that are independent and dependent on exogenous iron. We are not able to exclude the possibility that the exogenous iron-dependent portion is associated with loosely bound iron that is removed by chelating resin or by washing hepatocytes. In the present study, 50 μM Mn^{2+} inhibited lipid peroxidation in the presence of TBH by about 75%, while 50 μM Co^{2+} had no effect on lipid peroxidation. Since iron-dependent and -independent lipid peroxidation were of equal magnitude, the results cannot be explained entirely by the competition of manganese with putative loosely bound iron. In fact, manganese may inhibit lipid peroxidation by multiple mechanisms, such as reduction of lipid radicals formed during initiation and propagation [22, 29], and competion with iron binding sites [30]. The inhibitory effects of cobalt are less well understood. Inhibition by cobalt of lipid peroxidation in liposomes and rat liver microsomes has been ascribed to competition with iron for anionic oxygen on phosphate groups of phospholipids [31].

In conclusion, we have identified a redox active hepatocellular iron binding site that participates in hydroperoxide-mediated toxicity. Iron/hydroperoxide-mediated toxicity is not associated with lipid peroxidation under these assay conditions. The nature of this iron binding site and the critical toxicological target(s) are unknown.

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