Iron Supplementation Generates Hydroxyl Radical In Vivo

An ESR Spin-trapping Investigation

Maria B. Kadiiska,* Mark J. Burkitt,‡ Qun-Hui Xiang,§ and Ronald P. Mason*

**National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709; ‡Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen AB2 95B, United Kingdom; and §Department of Hygiene Chemistry, Faculty of Preventive Medicine, Hunan Medical University, Changsha Hunan 410078, China*

Abstract

Electron spin resonance (ESR) spectroscopy has been used to investigate hydroxyl radical generation in rats with chronic dietary iron loading. A secondary radical spin-trapping technique was used where hydroxyl radical forms methyl radical upon reaction with DMSO. The methyl radical was then detected by ESR spectroscopy as its adduct with the spin trap α-phenyl-*N*-*t*-butylnitrone (PBN). This adduct was detected in the bile of rats 10 wk after being fed an iron-loading diet and 40 min after the i.p. injection of the spin trap PBN dissolved in DMSO. Bile samples were collected into a solution of the ferrous stabilizing chelator 2,2′-dipyridyl in order to prevent the generation of radical adducts ex vivo during bile collection. Identification of the ESR spectrum of the major radical adduct as that of PBN/$^{\cdot}CH_3$ provides evidence for the generation of the hydroxyl radical during iron supplementation. Desferal completely inhibited in vivo hydroxyl radical generation stimulated by high dietary iron intake. No radical adducts were detected in rats which were fed the control diet for the same period of time. This is the first evidence of hydroxyl radical generation in chronic iron-loaded rats. (*J. Clin. Invest.* 1995. 96:1653–1657.) Key words: free radical • PBN • DMSO • iron diet • rats

Introduction

Clinical evidence for toxicity caused by chronic iron overload is now well established and is associated mainly with hepatocellular damage, fibrosis, and cirrhosis (1, 2). In humans, several disorders can lead to excess tissue-iron deposition, including hereditary hemochromatosis and various causes of secondary hemochromatosis such as thalassemia major, sideroblastic anemia, alcoholic cirrhosis, African dietary iron overload, transfusional iron overload, and excessive oral intake of iron (3, 4). In studies of patients with these disorders, a correlation has been demonstrated between the hepatic iron concentration and the occurrence of liver injury (5–7). In addition to the known association of chronic iron overload with the development of hepatic fibrosis and cirrhosis, there is now sufficient clinical evidence implicating iron overload in hepatocellular carcinoma (5).

Despite convincing clinical evidence for liver injury as a result of excess iron, the specific pathophysiological mechanisms for hepatocellular fibrosis, cirrhosis and carcinoma in iron overload are poorly understood. Iron within the liver is found in several biochemical forms such as ferritin, hemosiderin, heme, and also in the putative intracellular low molecular weight form chelated to nucleotides, amino acids and other low molecular weight compounds (8–10). Various theories of cellular toxicity resulting from excess iron deposition emphasize specific cytopathologic mechanisms whereby chronic iron overload causes organelle damage and altered cell function (4, 11). It has been suggested that excess iron deposition may cause cell death as a result of membrane lipid peroxidation both initiated and perpetuated by some form of intracellular iron (11–14). Peroxidative injury to isolated hepatic lysosomes, mitochondria, and microsomes has been found in animal models with chronic dietary iron overload (15–18).

Other mechanisms for iron-induced damage due to continuous overload have also been considered as potentially responsible for hepatic damage and injuries to other tissues (19). For example, iron-stimulated collagen biosynthesis is proposed as a possible explanation for fibrosis that does not necessarily require prior iron-mediated cellular damage (20). Iron induced damage to nucleic acids can lead to neoplasia (21). However, these postulated explanations for iron-induced injury are not mutually exclusive, and tissue damage may well be the result of multiple mechanisms (4).

In addition, the involvement of free radicals that damage critical cellular components has been implicated in the pathogenic process (4). The reactions leading to the production of oxidants that initiate oxidative damage to DNA, proteins and lipids have been extensively studied and reviewed (22, 23). Britton *et al.* (24) have shown an increase in a catalytically active pool of low molecular weight iron chelates in the livers of rats fed diets supplemented with carbonyl iron. Presumably, during continuous iron overload, the ability of the hepatocyte to maintain iron in nontoxic storage forms is exceeded, resulting in excessive amounts of low molecular weight iron chelate that is responsible for the generation of free radicals (4). There is now convincing evidence that the hydroxyl radical produced either by the Fenton reaction or by the iron-catalyzed Haber-Weiss reaction may be the species responsible for the damaging effects of iron (25).

Address correspondence to Maria B. Kadiiska, Ph.D., MD 10-02, National Institute of Environmental Health Sciences, NIH, P.O. Box 12233, Research Triangle Park, NC 27709. Phone: 919-541-0201; FAX: 919-541-7880.

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We have already reported the detection of in vivo–formed free radical species in rats treated acutely with high doses of iron (26). As already reported, the spin-trapping technique for the in vivo detection of hydroxyl radical adduct utilizes the reaction in which the hydroxyl radical is converted to the methyl radical ($^{\bullet}CH_3$) via its reaction with DMSO. The methyl radical is then detected as its adduct with the spin trap α-phenyl-*N*-*t*-butylnitrone (PBN)[1] by using electron spin resonance (ESR) spectroscopy (26). The data presented here extend the previous studies on hydroxyl radical formation generated by intragastric injection of ferrous salt. We have now examined the possibility of the generation of hydroxyl radical from rats with chronic dietary iron overload. This is the first ESR report of direct evidence for the induction of radical generation by continuous dietary iron loading.

Methods

PBN and dimethyl sulfoxide (DMSO) were purchased from Aldrich Chemical Co. (Milwaukee, WI). 2,2′-dipyridyl (DP) and deferoxamine mesylate (Desferal) were from Sigma Chemical Co. (St. Louis, MO) and $[^{13}C]_2$-dimethyl sulfoxide ($[^{13}C]_2$-DMSO) minimum 99% ^{13}C was from Isotec Inc. (Miamisburg, OH).

Sprague-Dawley male rats (Charles River Breeding Laboratories, Raleigh, NC) were used in all experiments. Rats were fed an iron-loaded Custom AIN-76A (ICN Biomedicals, Inc., Aurora, OH) diet modified with 1255 mg/kg (22.5 mmol/kg) iron as ferric citrate. Control rats were fed the same diet but with 35 mg/kg iron as ferric citrate. Another control group was fed the Custom AIN-76A diet modified with increased potassium and calcium citrate (22.5 mmol/kg) without additional iron to represent the dietary level of citrate in an iron-loaded diet. Diets and water were provided *ad libitum* for 10 wk.

At the end of the 10-wk diet period, rats were anesthetized (50 mg/kg body wt Nembutal) and bile ducts were cannulated with a segment of PE 10 tubing (Becton Dickinson & Co., Sparks, MD). All animals were given an i.p injection of the spin trap PBN (70 mg/kg or 250 mg/kg where indicated) dissolved in DMSO or in $[^{13}C_2]$DMSO (1 ml/kg). In some experiments Desferal was concomitantly administered i.p. at a dose of 1 mmol/kg.

Bile samples ($\sim$ 400 μl) were collected every 20 min for 2 h into plastic Eppendorf tubes containing 25 μl of 30 mM 2,2′-DP per 100 grams of rat body weight. Bile samples were frozen on dry ice immediately after collection. All spectra shown are from samples collected 20 to 40 min after the administration of PBN.

For in vitro experiments, bile from control or high-iron diet rats was added to tubes containing different concentrations of PBN, DMSO, DP, and ferric citrate.

ESR spectra were recorded on a Varian E-109 spectrometer with instrumental conditions indicated in the figure legends. Spectra were recorded on an IBM-compatible computer interfaced to the spectrometer. Hyperfine coupling constants were determined from a spectral simulation program (27).

Serum activities of lactic dehydrogenase (LDH), alanine aminotransferase (ALT), alkaline phosphatase (ALP), sorbitol dehydrogenase (SDH), and 5′ nucleotidase (5′-NT) and the serum concentration of total bile acids (TBA) were measured according to published procedures (28–31). Serum iron concentration and total iron-binding capacity (TIBC) were measured with the sample preparation kits IL Test Iron and IL Test TIBC, respectively, purchased from Instrumentation Laboratory (Lexington, MA) and performed at NIEHS in the Laboratory of Experimental Pathology. For all experiments in which the results of the group fed high-iron diet were compared with controls, an analysis of variance was applied. Values of $P \leq 0.05$ were taken to indicate significant difference.

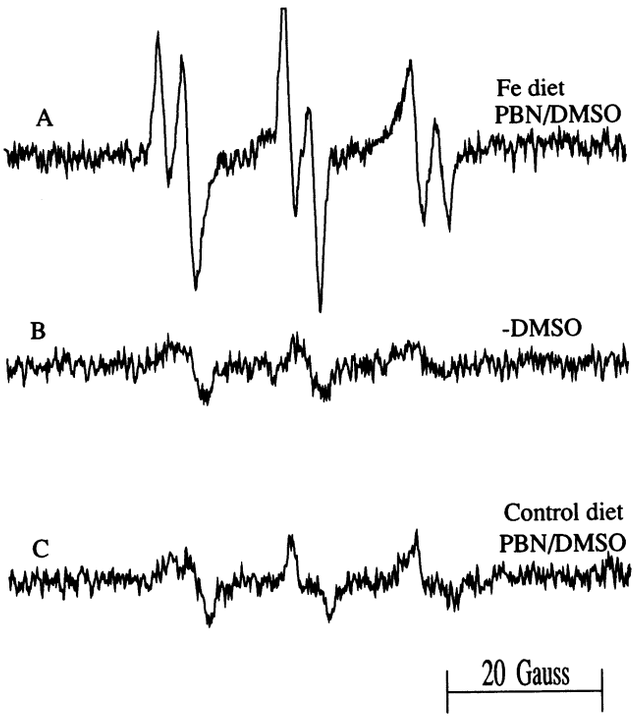

Figure 1. (*A*) ESR spectrum of radical adducts detected in the bile of rats fed the high-iron diet for 10 weeks collected 40 min after intraperitoneal administration of PBN (70 mg/kg) dissolved in DMSO (1 ml/kg). Bile samples ($\sim$ 400 μl) were collected into a 30 mM 2,2′-DP (25 μl/100g of rat weight). (*B*) Same as in *A*, but rats were not administered DMSO. (*C*) Same as in *A*, but rats were fed the regular control diet. Instrumental conditions: microwave power, 20 mW; modulation amplitude, 1.33 G; time constant, 1 s; scan time, 16 min.

Results

We detected the ESR spectrum of PBN radical adducts in the bile of rats fed the high-iron diet for 10 wk and collected 20 to 40 min after they had been dosed with PBN (Fig. 1 *A*). Essentially the same spectrum could also be detected 2 h after the injection of PBN with no statistically significant change in radical adduct concentration over this time period (data not shown). The radical adduct was also detectable in bile collected during the 0 to 20 min time interval, but the spectrum intensity was lower. As has been previously shown (26), the collection of samples into solution of 2,2′-DP was necessary in order to prevent ex vivo radical formation. Therefore, the prominent six-line ESR signal was detected from bile of iron-supplemented rats collected directly into 2,2′-DP solution. The ESR signal composition and intensity were completely altered when the experiment was repeated without DMSO (Fig. 1 *B*), thereby confirming the DMSO-dependence of radical formation. When we repeated the experiment, but with rats fed the control diet, only a residual PBN radical adduct signal was detected (Fig. 1 *C*). The same residual signal was detected from control rats that were fed the high citrate diet (data not shown). Since there was no significant ESR signal with either control group, all

1. *Abbreviations used in this paper:* ESR, electron spin resonance; PBN, α-phenyl-*N*-*t*-butylnitrone.

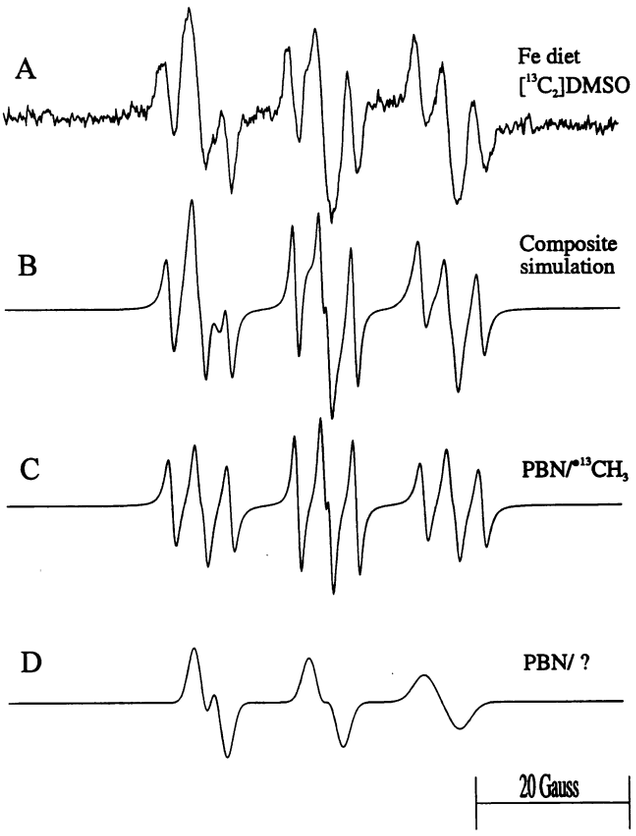

Figure 2. (*A*) ESR spectrum of radical adducts detected in the bile of rats fed the high-iron diet for 10 wk collected 40 min after intraperitoneal administration of PBN (70 mg/kg) dissolved in $[^{13}C]_2$DMSO (1 ml/kg). Bile samples (~ 400 μl) were collected into 30 mM 2,2′-DP (25 μl/100 grams of rat weight). (*B*) Complete computer simulation of the spectrum in *A*. (*C*) Simulation of the relative contribution of the PBN/$^{\bullet 13}CH_3$ radical adduct to the spectrum in *A*. (*D*) Simulation of the relative contribution of the unidentified PBN/? radical adduct to the spectrum in *A*. Instrumental conditions: microwave power, 20 mW; modulation amplitude, 1.33 G; time constant, 2 s; scan time 30 min.

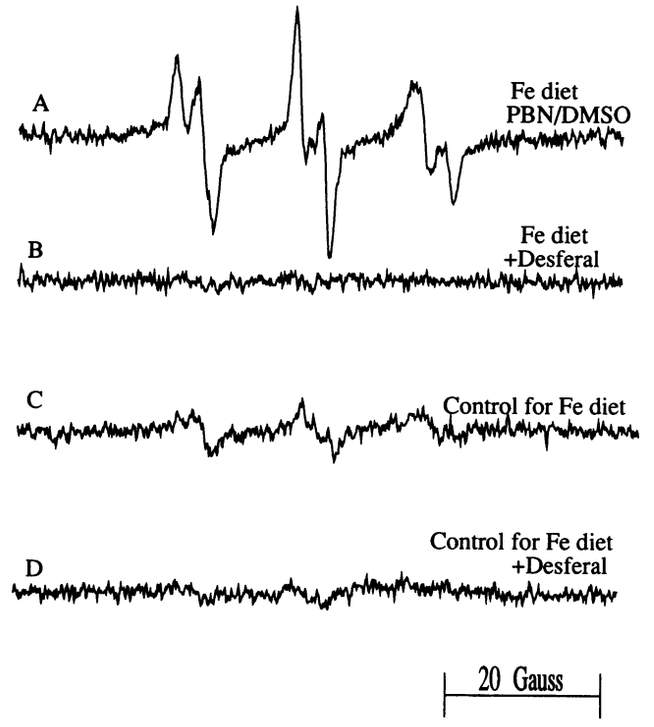

Figure 3. Effect of Desferal treatment on spectra detected in bile from rats fed high-iron and control diets. (*A*) ESR spectrum of radical adducts detected in the bile of rats fed the high-iron diet for 10 wk and 2 h after administration of PBN (70 mg/kg) dissolved in DMSO (1 ml/kg). Bile samples (~ 400 μl) were collected into 30 mM 2,2′-DP (25 μl/100 grams of rat weight). (*B*) Same as in *A*, but rats were also injected with Desferal (2 mmol/kg) intraperitoneally. (*C*) Same as in *A* but bile samples were from rats fed the regular control diet. (*D*) Same as in *C*, but rats were given Desferal (2 mmol/kg) intraperitoneally. Instrumental conditions were the same as in Fig. 1.

ESR control spectra are from rats that were fed control diets without high citrate.

As shown in Fig. 2 *A*, when we performed the in vivo experiment employing ^{13}C-labeled DMSO in place of isotopically normal DMSO, a characteristic 12-line spectrum from the PBN/$^{\bullet 13}CH_3$ adduct was detected. The appearance of ^{13}C-hyperfine splittings observed in the wings of each of the lines of the nitroxide triplet is proof that the radical adduct was derived from DMSO and cannot be derived from any other carbon source. The presence of another radical adduct was found during the simulation of the spectrum in Fig. 2 *B*. The major radical adduct with hyperfine coupling constants a^N = 16.36 G a^H_β = 3.36 G, and a^{13c}_β = 4.19 G was assigned to the PBN/$^{\bullet 13}CH_3$ based upon comparison of these splitting constants with those reported (26, 32, 33). The other unidentified PBN radical adduct had hyperfine coupling constants a^N = 15.03 G and a^H_β = 2.26 G and could not be identified. It is possible that this radical adduct is formed by the trapping of lipid-derived radicals, which have been detected from iron-supplemented hepatocytes (33 and references therein).

In order to investigate further the role of iron in the generation of hydroxyl radical in rats fed the high-iron diet, additional experiments were carried out in which animals were treated with an iron-chelating agent. Since Desferal is frequently found to inhibit iron-dependent hydroxyl radical formation and associated oxidative damage, and is used clinically in the treatment of certain iron overload conditions (3, 34, 35), it was chosen for this study. As shown in Fig. 3 the signal from the PBN/$^{\bullet}CH_3$ radical adduct detected from rats fed the high-iron diet (Fig. 3 *A*) was not detected when the animals were given Desferal (Fig. 3 *B*). Fig. 3, *C* and *D* show spectra from the bile of animals treated as in Fig. 3, *A* and *B* but fed regular control diet. No significant ESR signal was detected, thus confirming that the adduct in Fig. 3 *A* is dependent on the high-iron diet.

In addition, when ferric citrate (0.1 mM) was added in vitro to bile from non-treated rats containing PBN (10mM) in DMSO (10 mM) and DP (10 mM), only an ESR signal from the ascorbate anion radical could be detected (spectrum not shown). This finding demonstrates that the signal detected in the bile of rats fed the high-iron diet must be produced in vivo before sample collection.

We measured the activities of hepatic enzymes in blood serum to assess whether rats chronically fed the high-iron diet exhibit liver damage. We also measured total iron concentration and total iron-binding capacity in the blood serum of the same rats. It was found that there were no statistically significant

Table I. Effect of Iron Supplementation on the Rat Liver Enzyme Activities, Serum Iron and Total Iron-binding Capacity

	Control	*n*	High-iron diet	*n*	*P*
ALP (IU/liter)	75.5±5.3	10	79.6±5.1	9	NS
LDH (IU/liter)	1694±205	10	2265±321	9	NS
SDH (IU/liter)	21.1±3.6	8	24.2±2.8	9	NS
5′-NT (IU/liter)	34.7±2.7	10	35.2±2.9	9	NS
TBA (μmol/liter)	21.9±2.4	10	17.3±1.0	9	NS
ALT (IU/liter)	52.0±14.9	8	53.4±5.6	7	NS
Fe (μg/dl)	178.7±16.6	10	282.3±18.9	9	< 0.001
TIBC (μg/dl)	613.3±29.3	10	538.1±24.5	9	NS

ALP, alkaline phosphatase; LDH, lactic dehydrogenase; SDH, sorbitol dehydrogenase; 5′-NT, 5′nucleotidase; TBA, total bile acids; ALT, alanine aminotransferase; TIBC, total iron-binding capacity. Values are means±SE; NS, not significant; *n*, number of animals in each group.

differences in the serum enzyme activities studied between the control group and the rats fed the high-iron diet (Table I). However, there was statistically significant elevation of total iron and a decrease (not statistically significant, $P < 0.1$) in the total iron-binding capacity of blood serum in rats fed the high-iron diet as compared to the control group (Table I). Using plasma emission spectroscopy, light microscopy, or electron microscopy, no higher iron levels were found in the liver or in the bile of rats fed the high-iron diet (data not shown).

Discussion

The results in this study are the first to demonstrate in vivo hydroxyl radical generation in the conditions of dietary long term iron overload. Since the consequences of the various forms of iron overload are ultimately similar, the data presented here provide further evidence for the role of hydroxyl radicals in iron-induced injury.

The ESR evidence for hydroxyl radical formation in vivo due to the 10-wk dietary ferric citrate overload is provided by spin-trapping of the methyl radical resulting from the scavenging of hydroxyl radical by DMSO. Although ferric citrate complex has been ascribed a weak superoxide dismutase-like activity (36), it is also a redox active form of iron that can participate in Fenton chemistry and lipid peroxidation (37–39). The proposed mechanism for the reaction in vitro involves initiation by autoreduction of ferric citrate with subsequent autooxidation of the resulting ferrous ions (39–43). The detection of the DMSO-derived PBN/$^{\bullet}CH_3$ radical adduct in the bile of rats fed a continuously high ferric citrate diet suggests the generation of hydroxyl radical in ferric citrate toxicity. However, the evaluation of the detailed mechanism(s) in a whole animal is very complex. Among the unknowns are the chelators of the iron in vivo. In an animal model of acute iron overload, we have previously demonstrated that hydroxyl radical is generated and arises by the reduction of molecular oxygen to the hydroxyl radical via hydrogen peroxide without the requirement for pre-existing peroxides (26). Numerous studies and reviews on the pathology of chronic iron overload disorders suggested a role for iron-induced lipid peroxidation with consequent membrane damage, perhaps due to free radical formation (3, 12, 15, 44). Apparently, hydroxyl radical does not participate in the initiation of lipid peroxidation (45). The results in this study support literature data that indicate the ability of iron complexes including ferric citrate to catalyze hydroxyl radical generation (46, 47). The role of iron-chelation by citrate in the diet of these rats cannot be determined from this study. Additional experimental studies will be necessary to determine any effect of the various iron chelators as they relate to radical production in vivo.

Further evidence for the role of continuous dietary iron intake in the generation of hydroxyl radical has been provided by our observations of the effect of Desferal on hydroxyl radical generation. The finding that Desferal completely inhibits hydroxyl radical generation stimulated by high dietary iron intake provides strong evidence for the role of iron-dependent hydroxyl radical generation. Indeed, Desferal is reported to be effective in both clinical and experimental conditions of iron overload (3, 11).

The radicals detected in the bile from rats fed the high-iron-supplemented diet are presumably produced and trapped by PBN in the liver and excreted into bile. Indeed, we have demonstrated by various control experiments with rats fed a regular iron-supplemented diet or a high-citrate-supplemented diet that the radical adduct formation was dependent on supplementation with high ferric citrate. In vitro reactions of ferric citrate in bile confirmed that the radical adduct detected was formed in vivo and not in vitro during sample collection or handling.

After 10 wk on the high-iron–supplemented diet, iron deposition in the liver or in the bile was not found to be higher than in rats fed the regular-supplemented diet. From these experiments we conclude that free radical generation occurred in rats when the hepatic iron concentration was relatively low and not detectable with light microscopy, electron microscopy, or plasma emission spectroscopy. In addition, absence of statistically significant changes in the activities of hepatic enzymes proved lack of detectable liver damage in the conditions of our experimental model of chronic iron overload. In contrast, increased iron (280 versus 180 μg/dl) was measured in the blood serum of rats fed the high-iron diet. This study showed that hydroxyl free radical generation preceded liver damage and occurred concomitantly with modest increases in the total serum iron. Salonen *et al.* (48) reported significantly greater risk (2.2-fold) of acute myocardial infarct in men who had serum ferritin concentrations above 200 μg/dl, suggesting that such increase in iron loading can be clinically important. The recent proposal that low density lipoprotein peroxidation initiates the development of atherosclerosis (49) provides a possible link between high serum iron and increased risk of myocardial infarct because the hydroxyl radical we have detected will initiate lipid peroxidation.

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