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Direct evidence for *in vivo* hydroxyl-radical generation in experimental iron overload: An ESR spin-trapping investigation

(iron poisoning/free radicals/iron autoxidation)

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ABSTRACT **Although the hydroxyl radical is often implicated as the species responsible for the initiation of oxidative damage in iron-overload conditions, no ESR evidence for the formation of the radical *in vivo* has been reported. We have employed a secondary radical-trapping technique in which the hydroxyl radical reacts with dimethyl sulfoxide to form the methyl radical, which is then detected as its adduct of the spin trap *N*-*t*-butyl-α-phenylnitrone in the bile of animals given an intragastric dose of ferrous sulfate. The identity of this adduct was verified by isotope-substitution techniques. We show that unless measures are taken to inactivate the iron excreted in the bile of treated animals, reactions between iron, oxygen, dimethyl sulfoxide, *N*-*t*-butyl-α-phenylnitrone, and bile components lead to the formation of artifacts during sample collection.**

Although iron is an essential nutrient, the pathological processes associated with the various forms of iron overload demonstrate that the metal can also be toxic. Iron poisoning may be either acute or chronic. Acute iron poisoning is, in general, restricted to young children following the accidental ingestion of oral iron preparations (1). Chronic iron overload, however, has several causes, including excessive dietary intake (2), genetic hemochromatosis (3), and transfusional hemosiderosis (4).

Several animal-model studies into the pathology of iron indicate that oxidative damage to the membranes of cell organelles may be a crucial event in toxicity (5–9). In addition, a large body of evidence from *in vitro* studies involving isolated organelles (6, 10–12), cells (13), and tissue homogenates (14) suggests that oxidative damage is responsible for the toxic effects of iron.

Oxidative damage may be initiated by the hydroxyl radical (15), ·OH, generated in the reaction of ferrous iron with hydrogen peroxide, the Fenton reaction (16).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^- \quad [1]$$

In mammals, iron that is not incorporated into iron-utilizing proteins is rendered largely, or more likely, entirely, unavailable for participation in free-radical reactions by sequestration in storage or transport proteins (17). However, there is believed to exist a minute cellular pool of iron that is solubilized via chelation to low molecular weight biomolecules such as citrate and the adenine nucleotides (18–20). It is this intermediate pool of iron that is considered available for pathological free-radical reactions, which most likely occur to a significant extent only in conditions of iron overload.

Although superoxide is generated by a variety of enzymes [e.g., NADH dehydrogenase (21) and NADPH oxidase (22)], it is considered more likely that the reduced oxygen species proposed to be involved in iron toxicity arise via the direct reduction of molecular oxygen by iron (reactions 2, 3, and 1) (23).

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\cdot -} \quad [2]$$

$$Fe^{2+} + O_2^{\cdot -} + 2H^+ \rightarrow Fe^{3+} + H_2O_2 \quad [3]$$

Although many studies (5–12) have implicated ·OH in iron toxicity, no direct evidence in support of this proposal has been reported. In one study, ·OH production (determined as ethylene generation from 2-keto-4-methylthiobutyric acid) was demonstrated in the stomachs of rats given an intragastric dose of ferrous sulfate and ascorbic acid (24). This finding, however, provides little evidence to suggest that the induction of *systemic* iron-overload leads to ·OH production.

The most direct technique available for the detection of reactive free radicals is electron spin resonance (ESR) spectroscopy, which, for use *in vivo*, requires a spin-trapping approach (25, 26). With this approach, a radical, R·, that is too short-lived to detect by conventional ESR spectroscopy adds to a spin-trapping agent to form a relatively long-lived nitroxide radical adduct [see reaction 4 for the spin trap *N*-*t*-butyl-α-phenylnitrone (also called phenyl *N*-*t*-butylnitrone, PBN)]. The ESR spectrum of the radical adduct can then be used to identify R·.

$$C_6H_5{-}CH{=}\overset{+}{N}(O^-){-}C(CH_3)_3 \xrightarrow{R\cdot} C_6H_5{-}\underset{H}{\overset{R}{C}}{-}N(O\cdot){-}C(CH_3)_3 \quad [4]$$

Unfortunately, the ·OH adducts of the spin traps currently available appear to be too unstable to be of use *in vivo*. Further, due to the possibility that a superoxide adduct can decompose to give a ·OH adduct (27), the detection of the ·OH adduct of a spin trap is, in itself, not necessarily indicative of trapping free ·OH. Consequently, no ESR evidence for *in vivo* ·OH production has been published, to our knowledge.

In this study, we have used a well-known reaction in which ·OH is converted into the methyl radical, ·CH_3, via its reaction with dimethyl sulfoxide (DMSO) ($k = 7 \times 10^9$ $M^{-1} \cdot s^{-1}$, reaction 5) (28, 29). The methyl radical is then detected as its PBN adduct (reaction 6);

$$(CH_3)_2SO + \cdot OH \rightarrow CH_3SO_2H + \cdot CH_3 \quad [5]$$

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Abbreviations: DMSO, dimethyl sulfoxide; PBN, phenyl *N*-*t*-butylnitrone.

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$$PBN + \cdot CH_3 \rightarrow PBN/\cdot CH_3 \quad [6]$$

Alone, DMSO is relatively nontoxic, with a 24-hr LD_{50} of 13.7 g/kg (intraperitoneal) in the rat (30), and is therefore an ideal reagent for the *in vivo* detection of ·OH. Because PBN radical adducts, such as the structurally similar PBN/·CCl_3 species, have been detected previously in the bile of living animals (31), we decided to employ this relatively noninvasive sampling technique to investigate hepatic free-radical formation during iron poisoning. Here we report ESR evidence for ·OH generation in acute iron poisoning.

MATERIALS AND METHODS

DMSO, $FeCl_3$, $FeSO_4 \cdot 7H_2O$, $Na_2S_2O_4$, trichloroacetic acid, and PBN were from Aldrich. Deferoxamine mesylate (desferrioxamine), 2,2′-dipyridyl, and H_2O_2 were from Sigma and [$^{13}C_2$]DMSO (minimum 99 atom % ^{13}C) was from Isotec (Miamisburg, OH).

Male Sprague–Dawley rats (Charles River Breeding Laboratories), fed a standard chow mix (NIH Open Formula, Zeigler Brothers, Gardner, PA), were used in all experiments.

***In Vitro* Fenton Reaction.** Reactions were initiated by addition of $FeSO_4$ (from a concentrated stock solution prepared freshly in N_2-purged water) to a tube containing PBN, DMSO (or [$^{13}C_2$]DMSO), sodium phosphate buffer (pH 7), and H_2O_2 to give the final reagent concentrations indicated. Reaction mixtures were then analyzed immediately by ESR spectroscopy.

***In Vivo* Studies.** Nonfasted rats (330–750 g) were anesthetized with Nembutal and their bile ducts were cannulated using ≈7 cm of PE10 tubing. Anesthesia was maintained throughout the experiments, which were initiated by intraperitoneal injection of DMSO (1 ml/kg of body weight) containing PBN (70 mg/ml), followed by the intragastric injection of 1 M $FeSO_4$ (3 ml/kg) (prepared immediately before use in N_2-purged water). Where indicated, DMSO was omitted and the PBN (70 mg/kg) was administered as an aqueous solution (26 mg/ml), or both PBN and DMSO were omitted.

In all experiments, 20-min bile samples were collected (for 2 hr) into plastic Eppendorf tubes containing, when indicated, 30 mM 2,2′-dipyridyl (initially 100 μl, but in later experiments standardized to 25 μl per 100-g rat). Samples were frozen immediately on dry ice and stored at −70°C until ESR analysis was performed. All spectra shown are from samples collected between 100 and 120 min after the administration of $FeSO_4$.

Determination of Iron in Bile Samples. Following the intragastric administration of 1 M $FeSO_4$ (3 ml/kg) to anesthetized rats, 20-min bile samples were collected and frozen on dry ice prior to analysis for iron. Three hundred microliters of a thawed bile sample was placed in an Eppendorf tube, to which 7.5 μl of 0.25 M $Na_2S_2O_4$, 22.5 μl of 0.12 M 2,2′-dipyridyl in ethanol, 15 μl of water, and 75 μl of 5% (wt/vol) trichloroacetic acid were added. After vortex mixing and microcentrifugation (2000 rpm for 5 min), the absorbance of samples at 524 nm was measured against a blank prepared exactly as above, except that bile was taken from untreated animals. Values were then converted to iron concentrations by using a standard curve. For the preparation of the standard curve, known amounts of $FeSO_4$ were added (in a volume of 15 μl) to 300-μl bile samples taken from untreated animals. Absorbance values were then determined following the addition of $Na_2S_2O_4$ and 2,2′-dipyridyl, but not water, as described above.

***In Vitro* Autoxidation Experiments in Bile.** Reactions were initiated by the addition of an aliquot of either $FeSO_4$ or $FeCl_3$ to a tube containing PBN, DMSO, bile from untreated animals and, when indicated, either desferrioxamine or 2,2′-dipyridyl. After mixing and a 5-min incubation, mixtures were transferred to a quartz flat-cell and analyzed by ESR spectroscopy.

ESR Spectroscopy. Spectra were recorded using a Varian E-109 spectrometer, equipped with a TM_{110} cavity, operating at 9.33 GHz, 20-mW power, and 100-kHz modulation frequency. Data for spectra that were to be computer-simulated were acquired using an IBM-compatible computer and were analyzed for the determination of hyperfine coupling constant values with a program, based on the iterative search technique (32), developed by D. Duling.

RESULTS

To determine the suitability of the methyl radical-trapping technique, initial experiments were performed using a model Fenton system. Following the generation of ·OH from Fe^{2+} and H_2O_2 (reaction 1) in the presence of DMSO and PBN, a prominent ESR signal was detected (Fig. 1*a*). This spectrum consists of signals from two adducts of PBN, the predominant of which is characterized by hyperfine coupling constants from the nitroxide nitrogen (a^N) and the β-hydrogen (a_β^H) nuclei of 16.65 G (1 G = 10^{-4} T) and 3.58 G, respectively. The corresponding values for the weaker signal are 15.23 G and 3.05 G, respectively.

When DMSO was replaced by [$^{13}C_2$]DMSO, further coupling was detected (Fig. 1*b*), indicating the trapping of a ^{13}C-centered radical, the [^{13}C]methyl adduct. When the concentration of [$^{13}C_2$]DMSO was decreased from 5 M (Fig. 1*b*) to 250 mM (Fig. 1*c*), the signal from the [^{13}C]methyl adduct was largely replaced by a six-line signal (a^N = 15.20 G and a_β^H = 3.40 G), believed to correspond to the weaker signal detected in the initial experiment (Fig. 1*a*). The failure to detect coupling to ^{13}C in the six-line signal (Fig. 1*c*) indicates that the trapped radical is not carbon-centered, and the relative increase in the intensity of this signal observed at the lower DMSO concentration suggests that the trapped species may be a secondary radical formed in further reactions of nontrapped [^{13}C]methyl radicals.

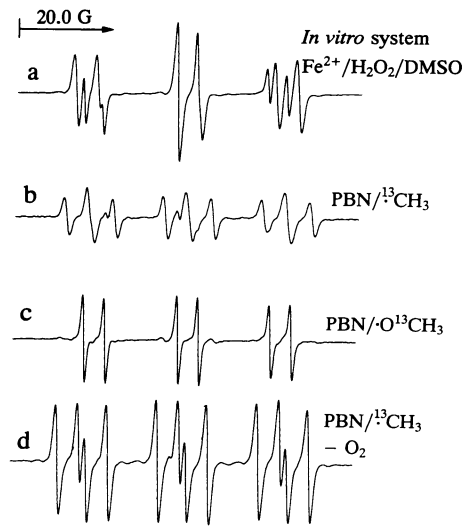

FIG. 1. Identification of radicals trapped by PBN during the oxidation of DMSO by ·OH. (*a*) Complete system, containing 50 mM PBN, 5 M DMSO, 2 mM H_2O_2, 50 mM sodium phosphate buffer (pH 7), and 2 mM $FeSO_4$ (added last). (*b*) Complete system, but using [$^{13}C_2$]DMSO. (*c*) As *b*, but with 0.25 M [$^{13}C_2$]DMSO. (*d*) As *c*, but the reagent solution was deoxygenated (by N_2 bubbling) prior to the addition of $FeSO_4$.

One secondary radical likely to be generated is the methoxy radical, $\cdot OCH_3$ (33), which would not be expected to form under anaerobic conditions (complementary oxygen-electrode experiments indicated that O_2 uptake occurs when $\cdot OH$ reacts with DMSO). Therefore, anaerobic incubations were performed using low concentrations of [$^{13}C_2$]DMSO. The spectrum of the [^{13}C]methyl adduct shown in Fig. 1*d* indicates that the removal of O_2 from incubations (by bubbling with N_2 prior to the addition of Fe^{2+}) prevents the formation of the adduct responsible for the six-line signal, thereby supporting the proposal that the adduct responsible for this signal (Fig. 1 *c* and *a*) is indeed that of the methoxy adduct to PBN. Due to the removal of oxygen, which causes line-broadening, the signal from the [^{13}C]methyl adduct shown in Fig. 1*d* shows greater resolution than that shown in Fig. 1*b*. The above spectral assignments, verified using ^{13}C substitution, are in agreement with the recent assignments of Britigan *et al.* (33) (Table 1).

The ESR analysis of bile samples taken from animals given an intraperitoneal injection of PBN in DMSO and an intragastric injection of $FeSO_4$ provided a prominent spectrum consisting of signals from more than one species. The dominant signal shows coupling to the nitrogen and β-hydrogen nuclei of *ca.* 16.3 G and 2.7 G, respectively (Fig. 2*a*). Although these values do not correspond exactly to those determined earlier for PBN/$\cdot CH_3$ or PBN/$\cdot OCH_3$ (Table 1), the generation of these adducts cannot be excluded, because the values shown in Table 1 are for specific solvent systems and therefore are not expected to be identical to those for the adducts in bile.

The weak signal detected in the bile of animals given PBN in DMSO alone may suggest the presence of a pool of either endogenous or contaminating iron capable of participating in radical reactions (Fig. 2*b*). Interestingly, when animals were administered PBN as an aqueous solution and iron, a strong signal was detected (Fig. 2*c*). Although this spectrum is poorly resolved, it is distinct from that observed when animals were given PBN in DMSO and iron (Fig. 2*a*), and is believed to result from the trapping of a radical from an endogenous source (e.g., a lipid). The administration of PBN alone provided only a barely detectable signal (Fig. 2*d*).

Before an attempt was made to identify unambiguously the radicals detected in these preliminary *in vivo* experiments, it was determined whether or not the adducts detected were formed within the animal or *ex vivo*, during collection. For example, excretion of the metal ion into the bile [a known route for iron excretion (34)], followed by its reactions with oxygen, DMSO, and bile components in the sample, may be responsible for the generation of the radical species detected. To investigate this possibility, the biliary excretion of iron

Table 1. Summary of hyperfine coupling constants (in Gauss) for the PBN adducts of radicals formed during the oxidation of DMSO by $\cdot OH$

Parent radical	a^N, G	a_β^H, G	a_β^{13C}, G	DMSO,* M	Ref.
$\cdot CH_3$	16.65	3.58		5.0	This work†
$\cdot CH_3$	16.51	3.68		0.14	33‡
$^{13}CH_3$	16.77	3.60	4.81	0.25	This work†
$^{13}CH_3$	16.26	3.40	4.40	ND	This work§
$\cdot OCH_3$	15.23	3.05		5.0	This work†
$\cdot O^{13}CH_3$	15.20	3.40		0.25	This work†
$\cdot OCH_3$	15.08	3.52		0.14	33‡

*The values of the hyperfine coupling constants were found to be affected by the concentration of DMSO.
†Radical generated as described in text and Fig. 1.
‡Radical generated using hypoxanthine, xanthine oxidase, and iron.
§Radical generated *in vivo* and detected in bile (see Fig. 5). Hence, the DMSO concentration was not determined (ND).

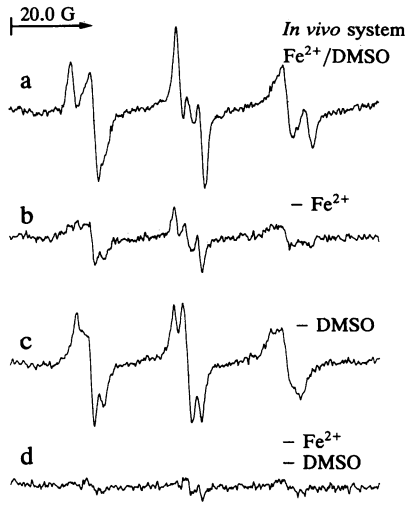

FIG. 2. Spectra of radical adducts detected in the bile from rats administered $FeSO_4$ (3 mmol/kg, intragastric) plus DMSO (1 ml/kg, intraperitoneal) containing PBN (70 mg/ml). (*a*) Complete system. (*b*) Complete system minus $FeSO_4$. (*c*) Complete system minus DMSO. (*d*) Complete system minus $FeSO_4$ and DMSO.

from treated animals was determined using 2,2′-dipyridyl. High concentrations of iron were detected in the bile samples (Table 2), suggesting that the radical adducts detected could indeed be generated *ex vivo*.

To investigate this possibility, experiments were performed in which reactants were added to bile from untreated animals. These experiments indicated that the addition of Fe^{2+} to bile containing PBN and DMSO results in the formation of PBN/$\cdot CH_3$. The adduct could also be detected when Fe^{2+} was replaced with Fe^{3+}, suggesting the presence of a reductant or Fe^{3+} in bile. This reactant may be either glutathione or ascorbate, both of which occur in bile. Therefore, in order to detect any radical adducts that are formed *in vivo*, precautions must be taken to prevent further, artifactual reaction from occurring during sample handling (e.g., the collection of bile into solutions of an iron-chelating agent). Further *in vitro* experiments showed that desferrioxamine inhibits the formation of PBN/$\cdot CH_3$ when reactions are initiated with Fe^{3+}, but not Fe^{2+}. This finding is consistent with the known ability of desferrioxamine to inhibit the reduction of Fe^{3+} and to promote the oxidation of Fe^{2+} (23). In contrast, the Fe^{2+}-stabilizing reagent 2,2′-dipyridyl was found to inhibit reactions initiated by both Fe^{2+} and Fe^{3+} (data not shown). Since iron excreted in the bile is expected to be maintained in the reduced state by glutathione and ascorbate, it was decided to collect samples directly into solutions of 2,2′-dipyridyl.

Table 2. Concentrations of 2,2′-dipyridyl-chelatable iron in the bile of rats following a single intragastric dose of $FeSO_4$

Sample collection time,* min	Biliary iron,† μM
0–20	–
20–40	10.0 ± 15.3
40–60	53.3 ± 5.1
60–80	100.8 ± 10.3
80–100	142.9 ± 40.5
100–120	153.3 ± 78.3

*Anesthetized rats were given an intragastric dose of $FeSO_4$ (3 mmol/kg). Bile samples were collected at the times indicated for the determination of dipyridyl-chelatable iron (see *Materials and Methods*).
†2,2′-Dipyridyl-chelatable iron in the presence of dithionite. Values are means ± SD (n = 3); –, not detectable.

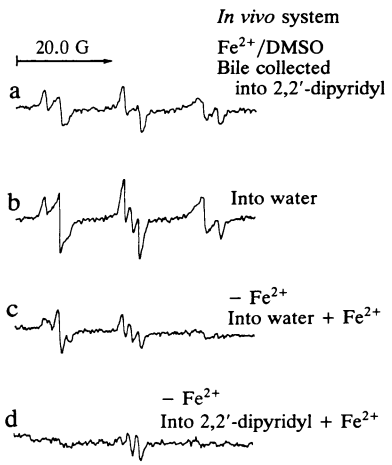

FIG. 3. Effects on spectra of bile collection into 2,2'-dipyridyl. Rats were administered $FeSO_4$ (3 mmol/kg, intragastric) plus DMSO (1 ml/kg, intraperitoneal) containing PBN (70 mg/ml). (*a*) Complete system, bile collected into 100 μl of 30 mM 2,2'-dipyridyl. (*b*) Complete system, bile collected into 100 μl of water. (*c*) Complete system, but rat was not given $FeSO_4$ and bile was collected into 100 μl of 2 mM $FeSO_4$. (*d*) Complete system, but rat was not given $FeSO_4$ and bile was collected into 100 μl of 30 mM 2,2'-dipyridyl/2 mM $FeSO_4$.

Following an intragastric dose of $FeSO_4$ and the intraperitoneal administration of PBN in DMSO, the ESR analysis of bile samples collected into a solution of 2,2'-dipyridyl provided a six-line spectrum (Fig. 3*a*). When samples were collected into an equivalent volume of water, however, a more intense signal was detected (Fig. 3*b*), suggesting that in the latter case some of the adduct detected had formed during sample handling. Indeed, when the bile from animals given only PBN and DMSO (no iron) was collected into tubes containing Fe^{2+} [final concentration *ca.* 400 μM, comparable to that detected in the bile from animals given a 3-mmol/kg oral dose of $FeSO_4$ (see Table 1)], a signal was detected (Fig. 3*c*) that was of lower intensity but otherwise identical to that detected in experiments in which the animals were given PBN, DMSO, and iron (with no 2,2'-dipyridyl in the collection tube) (Fig. 3*b*). To verify the ability of 2,2'-dipyridyl to prevent reactions occurring *ex vivo*, the bile from animals given only PBN and DMSO was collected into a solution of 2,2'-dipyridyl plus $FeSO_4$. The spectrum shown in Fig. 3*d* indicates that no detectable adduct is formed under such conditions (the weak doublet is from the ascorbyl radical),

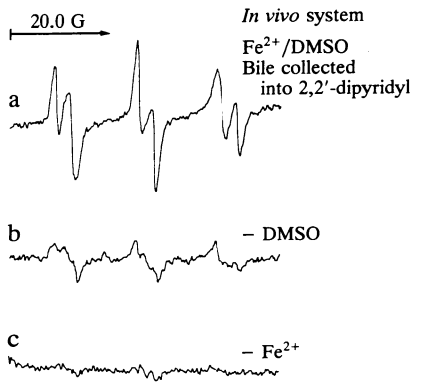

FIG. 4. DMSO and PBN dependence of *in vivo* radical-adduct formation. Rats were administered $FeSO_4$ (3 mmol/kg, intragastric) plus DMSO (1 ml/kg, intraperitoneal) containing PBN (70 mg/ml), and bile samples were collected into 100 μl of 30 mM 2,2'-dipyridyl. (*a*) Complete system. (*b*) Complete system minus DMSO. (*c*) Complete system minus $FeSO_4$.

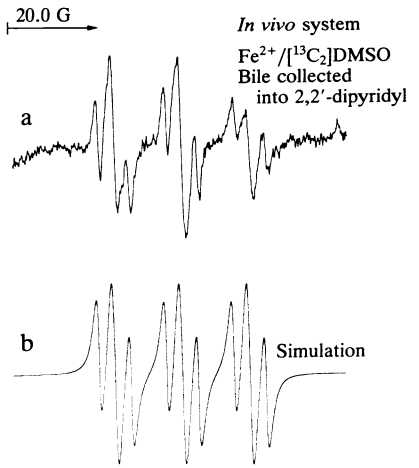

FIG. 5. Effect of ^{13}C substitution on spectra detected in the bile from radical adducts generated *in vivo*. (*a*) Rats were administered $FeSO_4$ (3 mmol/kg, intragastric) plus $[^{13}C_2]$DMSO (2 ml/kg, intraperitoneal) containing PBN (35 mg/ml). Bile samples were collected into 30 mM 2,2'-dipyridyl (25 μl per 100-g rat). (*b*) Computer simulation of *a* (coupling constants are given in text and Table 1).

thereby confirming that the signal shown in Fig. 3*a* is from an adduct formed *in vivo*.

The DMSO and iron dependence of *in vivo* adduct formation is demonstrated in Fig. 4. The weak signal detected in the absence of DMSO (Fig. 4*b*), which is dependent upon iron (Fig. 4*c*), indicates the trapping of a radical derived from an endogenous molecule. Other workers have also reported the trapping of endogenous radicals believed to be derived from lipids during membrane-lipid peroxidation (13, 14). When bile from a rat injected with PBN alone was collected without 2,2'-dipyridyl in the collection vessel, a weak signal was detected (see Fig. 2*d*), as described previously (31). Collecting bile into a solution of 2,2'-dipyridyl completely suppresses this weak signal, implying that it forms *ex vivo* from trace iron-catalyzed reactions.

Having demonstrated unambiguously *in vivo* iron-dependent free-radical formation, we next sought to identify the radical adduct(s) detected. Due to the unique solvent environment of a radical adduct immobilized in the biliary-micelle system, and due to the relatively small differences observed in the coupling constants of different PBN/radical adducts, the measurement of these values for an unknown adduct detected in such a system is not sufficient to provide unambiguous identification. Via the incorporation of an additional magnetic nucleus (typically ^{13}C or ^{17}O) into a radical adduct, isotope-substitution techniques have permitted the unambiguous identification of a variety of radicals trapped in complex biological systems (e.g., see ref. 26). Therefore, *in vivo* experiments were performed using $[^{13}C_2]$DMSO.

The ESR signal detected in the bile of rats given an intraperitoneal dose of PBN in $[^{13}C_2]$DMSO and an intragastric dose of $FeSO_4$ is shown in Fig. 5*a*. This spectrum is clearly dominated by a signal from the PBN/$^{13}CH_3$ radical adduct (*cf.* Fig. 1*b*) and was simulated using the hyperfine coupling constants $a^N = 16.26$ G, $a_\beta^H = 3.40$ G, and $a_\beta^{13C} = 4.40$ G (Fig. 5*b* and Table 1).

DISCUSSION

This study has provided ESR evidence for the generation of ·OH during acute iron poisoning. It is known from other studies, involving relatively well-defined chemical systems, that ·OH generation during iron(II) autoxidation involves the reduction of O_2 through H_2O_2 (refs. 23 and 35 and references therein). Therefore, we suggest that the ·OH radicals detected

in vivo during acute iron poisoning arise by the direct reduction of O_2 to ·OH via H_2O_2, without the requirement for preexisting endogenous peroxides.

The amount of iron administered in this study [3 mmol (0.83 g of $FeSO_4 \cdot 7H_2O$ per kg)] is well below the LD_{50} value [5.47 mmol (1.52 g) of oral $FeSO_4 \cdot 7H_2O$ per kg in mice (36)], indicating that free-radical generation may well be responsible for the toxicity of the agent at higher doses and that at lower, nonlethal doses, free-radical generation may produce changes at the molecular level (e.g., lipid peroxidation) responsible ultimately for the tissue damage associated with chronic iron poisoning. Indeed, the detection of a weak signal from animals administered iron and PBN alone provides evidence for the inducement of radical-mediated biomolecular oxidative damage during iron poisoning.

Because the scavenging of ·OH by DMSO leads to the formation of $\cdot CH_3$, which may in turn react with O_2 to yield reactive secondary radicals, DMSO cannot be considered to be a chain-terminating, antioxidant radical scavenger. Indeed, via its formation of reactive secondary radicals, DMSO may even promote the toxicity of ·OH. In contrast, due to the formation of relatively unreactive radical adducts, PBN might be expected to protect biological systems from the effects of free radicals. However, under the conditions employed here, it is considered unlikely that PBN or DMSO will react with all but a miniscule fraction of the ·OH radicals generated (due to competing reactions of ·OH with biomolecules), and therefore it is unlikely that either reagent will have a significant effect on toxicity due to radical scavenging.

Our investigations have also highlighted certain important methodological aspects that must be considered when iron-dependent free-radical reactions are investigated; in particular, attention must be given to the prevention of artifact formation during sample handling. For example, it is shown that the commonly used chelating agent desferrioxamine can be used to inhibit only reactions that are dependent on Fe^{3+} reduction (e.g., the Haber–Weiss cycle) but not those which depend on Fe^{2+} oxidation (e.g., iron autoxidation and the Fenton reaction). Therefore, care must be taken to ensure that, even in the presence of desferrioxamine, iron-dependent reactions do not occur during sample collection and handling (e.g., during tissue homogenization or bile collection). In this respect, further attention should be given to the potential use of chelating agents that stabilize iron in its 2+ oxidation state (e.g., 2,2′-dipyridyl and 1,10-phenanthroline) during such procedures.

In summary, our findings provide strong evidence for the role of ·OH in the toxicity of iron.

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