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Original Contribution

IRON AND DIOXYGEN CHEMISTRY IS AN IMPORTANT ROUTE TO INITIATION OF BIOLOGICAL FREE RADICAL OXIDATIONS: AN ELECTRON PARAMAGNETIC RESONANCE SPIN TRAPPING STUDY

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Abstract—Iron can be a detrimental catalyst in biological free radical oxidations. Because of the high physiological ratio of $[O_2]/[H_2O_2]$ ($\geq 10^3$), we hypothesize that the Fenton reaction with pre-existing H_2O_2 is only a minor initiator of free radical oxidations and that the major initiators of biological free radical oxidations are the oxidizing species formed by the reaction of Fe^{2+} with dioxygen. We have employed electron paramagnetic resonance spin trapping to examine this hypothesis. Free radical oxidation of: 1) chemical (ethanol, dimethyl sulfoxide); 2) biochemical (glucose, glyceraldehyde); and 3) cellular (L1210 murine leukemia cells) targets were examined when subjected to an aerobic Fenton ($Fe^{2+} + H_2O_2 + O_2$) or an aerobic ($Fe^{2+} + O_2$) system. As anticipated, the Fenton reaction initiates radical formation in all the above targets. Without pre-existing H_2O_2, however, Fe^{2+} and O_2 also induce substantial target radical formation. Under various experimental ratios of $[O_2]/[H_2O_2]$ (1-100 with $[O_2] \approx 250\ \mu M$), we compared the radical yield from the Fenton reaction vs. the radical yield from $Fe^{2+} + O_2$ reactions. When $[O_2]/[H_2O_2] < 10$, the Fenton reaction dominates target molecule radical formation; however, production of target-molecule radicals via the Fenton reaction is minor when $[O_2]/[H_2O_2] \geq 100$. Interestingly, when L1210 cells are the oxidation targets, $Fe^{2+} + O_2$ is observed to be responsible for formation of nearly all of the cell-derived radicals detected, no matter the ratio of $[O_2]/[H_2O_2]$. Our data demonstrate that when $[O_2]/[H_2O_2] \geq 100$, $Fe^{2+} + O_2$ chemistry is an important route to initiation of detrimental biological free radical oxidations.

Keywords—Electron paramagnetic resonance, Fenton reaction, Free radicals, Iron-oxygen complexes, Lipid peroxidation

INTRODUCTION

Iron is well known to be an important component of biological free radical oxidations; the mechanism that has been generally accepted for iron's participation involves both the Fenton and the iron-catalyzed Haber-Weiss reactions (Eqs. 1,2) [1–5].

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + HO^\bullet \quad \text{Fenton Reaction} \quad (1)$$

$$O_2^{\bullet -} + H_2O_2 \xrightarrow{\text{Iron}} O_2 + OH^- + HO^\bullet \quad \text{Haber-Weiss Reaction} \quad (2)$$

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An alternate mechanism, however, has been proposed: free radical oxidations, such as lipid peroxidation, are not necessarily initiated by $HO^\bullet$ formed from the Fenton or Haber-Weiss reactions, but rather, oxidations are initiated by iron in the form of 'Fe-O' complexes, i.e., perferryl or ferryl ions [6–11]. In this work we use 'Fe-O' to generically denote the iron-oxygen complexes that initiate oxidations. Although the oxidations are started with Fe^{2+} and O_2, the exact nature of the initiating species are unknown. Thus, we use '$Fe^{2+} + O_2$' to denote generically the chemistry and the species that initiate the observed oxidations.

The perferryl ion is an intermediate product that can be produced through either Fe^{2+}/O_2 or $Fe^{3+}/O_2^{\bullet -}$ (Eq. 3):

$$Fe^{2+} + O_2 \Leftrightarrow [Fe^{2+}\text{-}O_2 \Leftrightarrow Fe^{3+}\text{-}O_2^{\bullet -}] \Leftrightarrow Fe^{3+} + O_2^{\bullet -} \quad \text{Perferryl ion} \quad (3)$$

The ferryl ion is thought to be formed by two routes. The first relies on superoxide-dependent reduction of Fe^{3+} to Fe^{2+} and subsequent reaction of Fe^{2+} with H_2O_2. Under proper conditions of pH and polarity, Fenton reagents will produce ferryl ion rather than $HO^{\bullet}$ [2,6] (Eqs. 4 and 5):

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

$$Fe^{2+} + H_2O_2 \rightarrow \underset{\text{Ferryl ion}}{Fe^{2+}O} + H_2O \quad (5)$$

A second route to ferryl ion formation is by the reaction of perferryl ion with another Fe^{2+} [12] (Eqs. 6 and 7).

$$Fe^{2+} + \underset{\text{Perferryl ion}}{Fe^{2+}\text{-}O_2} \rightarrow Fe^{2+}\text{-}O_2\text{-}Fe^{2+} \quad (6)$$

$$Fe^{2+}\text{-}O_2\text{-}Fe^{2+} \rightarrow \underset{\text{Ferryl ion}}{2Fe^{2+}\text{-}O} \quad (7)$$

Due to their high electron affinities, perferryl and ferryl ions could be important oxidants in detrimental biological oxidations, with reactivities approaching that of $HO^{\bullet}$.

From a thermodynamic point of view, the highly oxidizing products of the Fenton and Haber Weiss reactions can easily initiate biological oxidations, including DNA, protein and lipid oxidation. However, there are several shortcomings for this route. First, the yield of $HO^{\bullet}$ in biological systems from the iron-catalyzed Haber-Weiss reaction will be extremely low because of the small rate constant of the Fenton reaction (about 10^3–10^5 M^{-1} s^{-1}) [13]. In addition the high rate constant (about 10^6–10^7 M^{-1} s^{-1}) for the oxidation of Fe^{2+} by $O_2^{\bullet -}$ ($Fe^{2+} + O_2^{\bullet -} \rightarrow H_2O_2 + Fe^{3+}$) at physiological conditions may limit the Fe^{2+} available [14,15]. Third, because of its high reactivity, $HO^{\bullet}$ has a limited lifetime in cells and tissues, about 1 ns [16]. Thus, it is difficult to conceive that $HO^{\bullet}$ migrates from sites of generation in the aqueous regions to hydrophobic membrane compartments where lipid peroxidation is triggered, without first reacting with other biomolecules. Finally, perhaps the most important reason to consider the oxidative chemistry initiated by $Fe^{2+} + O_2$ as a significant route to biological oxidations is that the overall steady-state concentration of oxygen is much greater than hydrogen peroxide in living systems [17–20].

In most regions of cells, $[H_2O_2]_{ss}$ (the subscript ss is used to designate a steady state concentration of a species) may be too low for the Fenton reaction to be of major significance. For example, $[H_2O_2]_{ss}$ in the red blood cell is estimated to be approximately 10^{-10} M [18]; in the inner mitochondrial membrane $[H_2O_2]_{ss}$ is estimated as 10^{-8} M [20]. Measurements in liver cells have determined the steady state level of H_2O_2 to be $\approx 10^{-8}$ M [17].

The rate of $HO^{\bullet}$ formation by the Fenton reaction with pre-existing H_2O_2 can be expressed as (Eq. 8):

$$\begin{aligned} \text{rate } (HO^{\bullet} \text{ formation}) &\approx k_{\text{Fenton}}[H_2O_2]_{ss}[Fe^{2+}] \\ &\approx (10^3\ M^{-1}s^{-1})(10^{-8}\ M)[Fe^{2+}] \\ &\approx 10^{-5}[Fe^{2+}]s^{-1} \end{aligned} \quad (8)$$

If each $HO^{\bullet}$ formed results in oxidation of biological material, then the rate of biological substrate oxidation will be (Eq. 9):

$$\text{rate (substrate oxidation)} \approx 10^{-5}[Fe^{2+}]s^{-1} \quad (9)$$

In vivo, however, $[O_2]_{ss}$ is about 10^{-5} M [19], much higher than $[H_2O_2]_{ss}$; dioxygen can react readily with $[Fe^{2+}]_{ss}$ forming species that can initiate biological oxidations. If we assume that the rate constant for oxidation of substrate by '$Fe^{2+} + O_2$' chemistry is similar to the Fenton reaction ($k_{\text{Fe-O2}} \approx k_{\text{Fenton}} \approx 10^3\ M^{-1}s^{-1}$) and that the oxidizable substrate concentration of a living system is about 1 M (in living systems the percentage by weight of oxidizable components [protein, lipid, and the bases of RNA and DNA] $\approx$30 %; from the molecular weight of their components, the oxidizable substrate concentration would be $\approx$1 M.), the oxidation rate by '$Fe^{2+} + O_2$' chemistry can be estimated as Eq. 10:

$$\begin{aligned} \text{rate (substrate oxidation)} &\approx k_{\text{Fe-O2}} \times \\ &\quad [\text{`}Fe^{2+} + O_2\text{'}]\text{oxidizable substrate}] \\ &\approx (10^3\ M^{-1}s^{-1})\ [\text{`}Fe^{2+} + O_2\text{'}]\ 1\ M) \\ &\approx 10^3[Fe^{2+}]\ s^{-1} \end{aligned} \quad (10)$$

This rate assumes that nearly all "loosely bound" or catalytic iron will be present as Fe^{2+}. Keyer and Imlay [22] have provided direct evidence supporting this assumption. In addition, we assume that this Fe^{2+} will readily form Fe^{2+}–O_2 complexes. This assumption is supported by the many published observations that Fe^{2+} can be very short-lived (seconds to minutes depending on conditions and coordination environment) in neutral, aerated solution [24,25].

Thus, 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. This 10^8 ratio of rates assumes that all Fe^{2+} results in '$Fe^{2+} + O_2$'. Clearly this would not be the case. However, if only 1/100 were to form this species the ratio of rates would still be 10^6. Furthermore, the '$Fe^{2+} + O_2$' hypothesis has additional strengths; for example, $O_2^{\bullet -}$ is not needed in this mechanism for the reduction of iron. Ascorbate, glutathione or other reducing agents (perhaps even reductase enzymes) present in biological systems can always serve as reductants for iron. The steady-state con-

centration of superoxide in cells is believed to be on the order of 10^{-10} M [17,21,22]. Thus, in these circumstances superoxide will be kinetically insignificant as a reductant of iron for the Fenton reaction and its oxidizing capability, via $HO_2^{\bullet}$, will most likely dominate [21–23].

To examine our hypothesis that the Fenton reaction with pre-existing H_2O_2 is only a minor initiator of biological oxidations compared to the 'Fe^{2+} + O_2' mechanism, we have experimentally compared the relative importance of oxidations initiated by the Fenton reaction with pre-existing H_2O_2 vs. oxidations initiated by Fe^{2+} and dioxygen chemistry. We used EPR spin trapping to study the oxidation of chemical, biochemical, and cellular targets under various ratios of $[O_2]/[H_2O_2]$. The results from the chemical and biochemical free radical oxidations examined suggest:

1. The importance of the Fenton reaction significantly decreases as $[O_2]/[H_2O_2]$ increases.
2. The Fenton reaction with pre-existing H_2O_2 is negligible compared with the chemistry of 'Fe^{2+} + O_2' at high ratios of $[O_2]/[H_2O_2]$, e.g., $\geq$ 100.

Furthermore, our cellular oxidation results show that pre-existing H_2O_2 does not enhance free radical oxidations initiated by iron, but rather in some circumstances, may inhibit cellular radical formation. We conclude that, under physiological conditions, the Fenton reaction with pre-existing H_2O_2 will usually have only a minor role in initiating biological oxidations and we suggest that iron-oxygen complexes can be the primary initiators of biological free radical oxidations.

EXPERIMENTAL PROCEDURES

Chemical reagents

All experiments were performed in 50 mM (pH 7.4) potassium phosphate-buffered aqueous solution. Adventitious metals were removed by treatment with chelating resin (sodium form, dry mesh 50–100, from Sigma, St. Louis, MO, USA) using the batch method; absence of metal was verified with the ascorbate test [26]. The spin trap POBN, α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (Oklahoma Medical Research Foundation Spin Trap Source, Oklahoma City, OK, USA) was prepared as a 1.0 M aqueous stock solution immediately before use and kept on ice during the experiment. Ferrous ammonium sulfate ($Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, Fisher Scientific Co., Fair Lawn, NJ, USA) was used to prepare 10 mM stock solutions of Fe^{2+} in redistilled water. The pH was controlled by HCl to keep it about 2.5. At this pH, Fe^{2+} is quite stable. H_2O_2 (30%, EM Science, Gibbstown, NJ, USA) was used to prepare 10 mM stock solutions using ϵ_{240} = 39.4 M^{-1} cm^{-1}, in redistilled water. DMSO (Sigma) and EtOH (100%, Fisher) were introduced directly into the oxidation systems as needed to make 100 mM solutions. About 3 μL DMSO and 3.5 μL EtOH neat solutions were required to prepare 500 μL EPR samples. Glucose (EM Science) 2.0 M and 1.0 M glyceraldehyde (EM Science) stock solutions were prepared with phosphate buffer immediately before introduction into the oxidation system to make a 200 mM glucose or a 100 mM glyceraldehyde solution. Ethylenediaminetetraacetic acid (EDTA) and DTPA (Sigma) were dissolved in redistilled water by moderate heating to make 10.0 mM stock solutions. When chelated-Fe^{2+} solution was used, the ratio of EDTA or DTPA/Fe^{2+} was 1.1/1. To keep iron as Fe^{2+}, N_2 or argon gas was used to remove oxygen. Catalase (CAT, Sigma), and superoxide dismutase (SOD, from bovine erythrocytes, Sigma) stock solutions were prepared with redistilled water and then introduced into the oxidation systems to make final concentrations of 500 U/mL for CAT and 50 U/mL (cytochrome c method) for SOD. To control for non-specific protein effects bovine serum albumin (BSA, Sigma) was used at the same protein concentration as the total protein in the SOD plus CAT experiments.

L1210 cells

L1210 murine leukemia cells were grown in a humidified atmosphere of 95% air–5% CO_2 at 37°C in RPMI 1640 (Grand Island Biochemical Co., Grand Island, NY, USA) medium containing 10% fetal bovine serum (Sigma). The cells were maintained as an exponentially growing culture by passage every 3 days. Cell lipids were modified by addition of 32 μM docosahexaenoic acid (DHA, Cayman Chemical Co., Ann Arbor, MI, USA) to the growth medium for 48 h before the radical experiments. Immediately before the EPR spin trapping experiments, cells were washed three times with phosphate buffer solution to remove all growth medium. Cells were resuspended in the phosphate buffer solution at a density of 5 $\times$ 10^6 cells/mL for the EPR measurements.

The order of addition

The order of addition of reaction reagents is an important factor that may affect the results. In our experiments, the target materials were usually mixed with phosphate buffer before introducing any other reagents. The spin trap POBN and then H_2O_2 were added to the system. The Fe^{2+} solution was always added to the oxidation system last.

Electron paramagnetic resonance measurements

All EPR spectra were obtained with a Bruker ESP-300 spectrometer (Karlesruhe, Germany) operating at 9.76 GHz

and room temperature. The EPR spectrometer settings were: modulation frequency 100 kHz; modulation amplitude 1.0 G; microwave power 40 mW; receiver gain 10^5–10^6. 3-Carboxy proxyl (Aldrich Chem Co, Milwaukee, WI, USA) was used as the standard for POBN radical adduct quantitation. Spin adduct yield was determined from the peak height or peak area of the central doublet.

Oxygen monitor

A YSI Model 5300 Biological Oxygen Monitor (Yellow Springs Instruments, Yellow Springs, OH, USA) was used to measure H_2O_2 from the oxygen yield when catalase was added to dismute H_2O_2 ($2\ H_2O_2 \rightarrow O_2 + 2\ H_2O$).

RESULTS AND DISCUSSSION

To examine the hypothesis that the Fenton reaction with pre-existing H_2O_2 is only a minor initiator of biological oxidations compared to the '$Fe^{2+} + O_2$' mechanism, we have experimentally compared the Fenton reaction efficiency vs. '$Fe^{2+} + O_2$' reaction efficiency in the initiation of oxidation of chemical, biochemical, and cellular targets under various $[O_2]/[H_2O_2]$ ratios. When DMSO, EtOH, glucose, glyceraldehyde or cultured L1210 murine leukemia cells are subjected to either a Fenton system or an $Fe^{2+} + O_2$ system, target-derived free radicals can be spin trapped by POBN (representative spectra are shown in Fig. 1). Carbon-centered radicals are spin trapped from all targets, e.g., $^{\bullet}CH_3$ from DMSO, and $CH_3 \cdot CHOH$ from EtOH. In the DMSO system, $^{\bullet}OCH_3$ is also trapped by POBN under aerobic conditions.

Oxygen and the apparent stoichiometry of radical formation

When oxidation targets are exposed to Fe^{2+} and H_2O_2 under anaerobic conditions, the Fenton reaction would be considered as a "pure" Fenton reaction. The stoichiometry of Fe^{2+} consumption vs. target radical formation should be representative of the authentic Fenton reaction. With excess target and H_2O_2, it would be 1:1 in accordance with the following reactions (Eqs. 11–13):

$$Fe^{2+} + H_2O_2 \longrightarrow OH^- + Fe^{3+} + HO^{\bullet} \quad (11)$$

$$Target + HO^{\bullet} \longrightarrow Target^{\bullet} + H_2O \quad (12)$$

$$Fe^{2+} + Target + H_2O_2 \longrightarrow Target^{\bullet} + OH^- + Fe^{3+} + H_2O \quad (13)$$

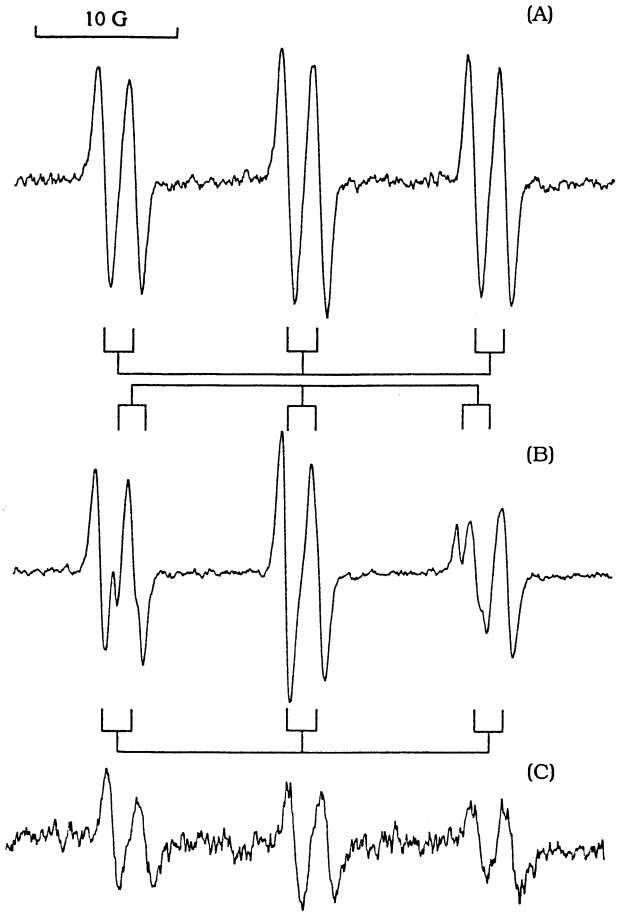

Fig. 1. Each target produces radicals as seen by EPR spin trapping using POBN when Fe^{2+} (100 μM) is introduced into a pH 7.4 phosphate-buffered solution of the target species. (A) This type of spectrum can be observed from EtOH, glucose, and glyceraldehyde aerobic oxidations and DMSO anaerobic oxidation. In these oxidations, only one carbon-centered radical from each target was trapped by POBN. The splitting constants of POBN adducts from these targets are: EtOH (a^N = 15.8 G; a^H = 2.7 G); glucose (a^N = 15.6 G; a^H = 2.4 G); glyceraldehyde: (a^N = 15.7 G; a^H = 2.7 G); and DMSO anaerobic oxidation (a^N = 16.1 G; a^H = 2.8 G). (B) This spectrum can be observed from DMSO aerobic oxidation. In addition to the carbon-centered radical trapped by POBN: a^N = 16.1 G and a^H = 2.8 G, the $^{\bullet}OCH_3$ radical is also observed during DMSO oxidation under aerobic conditions with (a^N = 14.8 G and a^H = 2.4 G) [27]. (C) This spectrum can be observed from PUFA-enriched L1210 oxidations. Based on the accepted lipid peroxidation mechanism and the EPR hyperfine splitting constants (a^N_1 = 15.5 G and a^H_1 = 2.9 G, a^N_2 = 15.8 G and a^H_2 = 2.5 G), we assigned these adducts to two POBN alkyl radical adducts that arise from fragmentation of the oxidized lipid chain [27,32,33].

Under aerobic conditions, if $HO^{\bullet}$ formed from the Fenton reaction is the only initiator of free radical oxidations, then this same 1:1 stoichiometry of Fe^{2+} consumption vs. target radical formation would be obtained. However, if $HO^{\bullet}$ is not the only initiator of free radical oxidations, then a different stoichiometry of Fe^{2+} consumption could be obtained. (Note, water is not a major product when the target is DMSO. The major products of DMSO oxidation by $HO^{\bullet}$ are $^{\bullet}CH_3$and $CH_3S(O)OH$.)

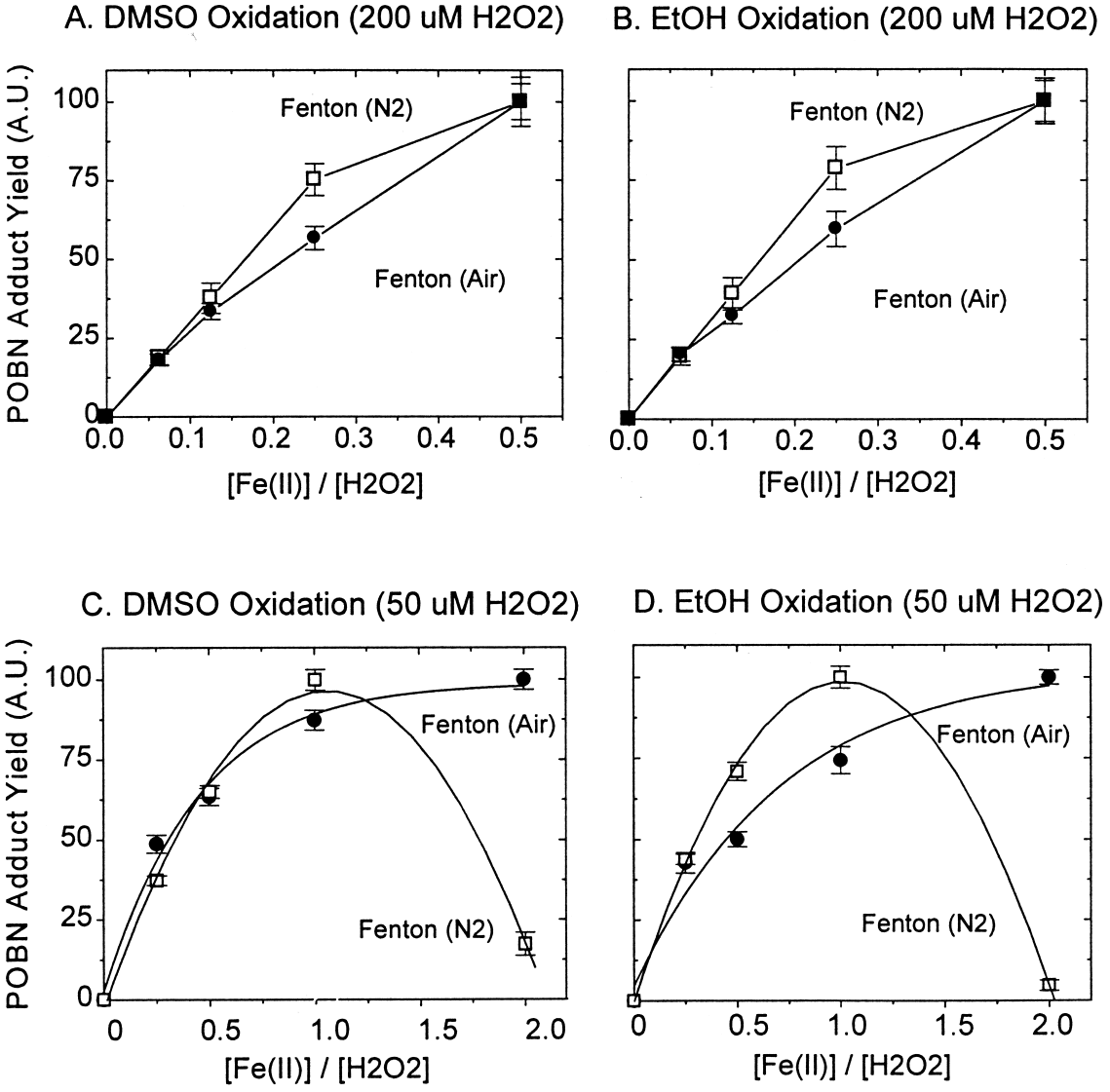

Fig. 2. Fe^{2+} consumption vs. POBN adduct yield is different in aerobic and anaerobic Fenton reactions. (A,B) DMSO (100 mM) and EtOH (100 mM) oxidized by variable [Fe^{2+}] and 200 μM H_2O_2 with/without oxygen present. The paired comparison of anaerobic and aerobic curves in DMSO and EtOH oxidations shows them to be statistically different, $p < .01$. One hundred A.U. in DMSO oxidation represents 0.79 μM POBN adduct for aerobic and 5.5 μM POBN adduct for anaerobic conditions. One hundred A.U. in EtOH oxidation represents 2.3 μM POBN adduct for aerobic and 7.3 μM POBN adduct for anaerobic conditions. (C,D) DMSO (100 mM) and EtOH (100 mM) oxidized by variable [Fe^{2+}] and 50 μM H_2O_2 with/without oxygen present. The paired comparison of anaerobic and aerobic curves in DMSO and EtOH oxidations shows the statistically different, $p < .001$. One hundred A.U. in DMSO oxidation represents 0.56 μM POBN adduct for aerobic and 3.9 μM POBN adduct for anaerobic conditions. One hundred A.U. in EtOH oxidation represents 1.2 μM POBN adduct for aerobic and 4.9 μM POBN adduct for anaerobic conditions.

We have used EPR spin trapping with POBN in both aerobic and anaerobic conditions to measure radical formation from targets oxidized via Fenton reactants ([Fe^{2+}] ranged from 0 to 100 μM with 50 μM or 200 μM H_2O_2). We assumed that: 1) Fe^{2+} is completely consumed by using excess H_2O_2 in both anaerobic and aerobic conditions; and that 2) total target radical formation is proportional to POBN adduct yield due to the relatively high POBN concentration and the following fast reaction (Eq. 14):

$$^{\bullet}\text{Target} + \text{POBN} \longrightarrow \text{POBN/Target radical adduct} \quad (14)$$

Indeed, under anaerobic conditions, the expected linear relationship for POBN adduct yield vs. [Fe^{2+}] is observed when [Fe^{2+}]/[H_2O_2] is low (Fig. 2A,B). But, under anaerobic conditions, when [Fe^{2+}]/[H_2O_2] is high (>1), the observed yield of POBN radical adduct is less

than the ideal (Fig. 2C,D). Two possible reactions that may contribute to this decrease in yield are (Eqs. 15 and 16):

$$Fe^{2+} + HO^{\bullet} \longrightarrow OH^{-} + Fe^{3+} \quad (15)$$

and

$$Fe^{2+} + \text{POBN/adduct} \longrightarrow \text{non radical product} \quad (16)$$

These results clearly demonstrate that under anaerobic conditions and low $[Fe^{2+}]$ the two assumptions above seem to hold. However, in the presence of O_2, high $[Fe^{2+}]$ always resulted in a high yield of POBN adduct when $[Fe^{2+}] > [H_2O_2]$ (Fig. 2). This suggests that the Fenton reaction may not be the only mechanism of initiation, but other initiators should be considered, such as 'Fe-O' complexes.

In the absence of oxidizable substrate, the ideal stoichiometry for the reaction of Fe^{2+} with H_2O_2 would be 2:1. $HO^{\bullet}$ can also react with H_2O_2 ($k = 3 \times 10^7$ M^{-1} s^{-1}), but the reaction with Fe^{2+} is rapid ($k = 4 \times 10^8$ M^{-1} s^{-1}) [28]. In our experiments the reaction with Fe^{2+} should dominate, see Eqs. 17–19:

$$Fe^{2+} + H_2O_2 \longrightarrow OH^{-} + Fe^{3+} + {}^{\bullet}OH \quad (17)$$

$$Fe^{2+} + HO^{\bullet} \longrightarrow OH^{-} + Fe^{3+} \quad (18)$$

$$2Fe^{2+} + H_2O_2 \longrightarrow 2\,OH^{-} + 2\,Fe^{3+} \quad (19)$$

Indeed, H_2O_2 depletion follows the expected stoichiometric ratio of Fe^{2+}/H_2O_2 (2:1) very well under anaerobic conditions, Table 1. However, in the presence of oxygen the ideal stoichiometry does not hold well when $[Fe^{2+}]$ is in excess of H_2O_2. This is consistent with the formation of 'Fe-O' complexes thereby sparing H_2O_2 as well as the subsequent formation of some H_2O_2 from $Fe^{2+} + O_2$ [29,30]. This different stoichiometry of H_2O_2 consumption in aerobic and anaerobic conditions suggests that '$Fe^{2+} + O_2$' chemistry may produce important oxidants that initiate free radical oxidations.

Pre-existing H_2O_2 is NOT necessary for radical formation

It has been our experience that all that is needed to initiate free radical oxidations in neutral solution is the simultaneous presence of Fe^{2+} (in an appropriate coordination environment) and dioxygen [31–33]. Indeed, the addition of Fe^{2+} to aerobic solutions of DMSO, EtOH, or glucose all give rise to target radicals as seen by POBN spin trapping, Fig. 1 and Table 2. In the experiments shown in Table 2 no H_2O_2 was added, thus the oxidations were initiated by species derived from Fe^{2+} and O_2. Fe^{2+} and O_2 can produce superoxide or H_2O_2 in appropriate environments [4,9,29,30]. To determine if these species have a role in the oxidation of these targets, SOD, CAT, or SOD plus CAT were added to the solution before introduction of Fe^{2+}. Superoxide dismutase was seen to inhibit the production of target radical by Fe^{2+} and dioxygen. Catalase had a minor inhibitory effect that did not always achieve statistical significance. However, the presence of both SOD and CAT seemed to produce an additive effect as seen by the inhibition of target radical formation; an approximate 50% reduction of target radical formation was achieved. These results clearly demonstrate that pre-existing hydrogen peroxide is not needed for Fe^{2+} to initiate oxidations when dioxygen is present and that SOD and CAT are able to protect from this damage. Because SOD and CAT are able to provide some protection, this suggests that $Fe^{2+} + O_2$ results in the formation of $O_2^{\bullet -}$ and H_2O_2 and that these species are partially involved in the oxidation of the target molecules.

Table 1. Observed stoichiometry of the Fenton reaction[a]

$[Fe^{2+}]/\mu M$	$[H_2O_2]_{Theory}$[b]	$-O_2$[c] $[H_2O_2]^{e}_{Exp}$ (μM)	$+O_2$[d] $[H_2O_2]_{Exp}$ (μM)
0	25	24.8 ± 0.6[f]	24.8 ± 0.5
25	12.5	13.7 ± 1.3	12.7 ± 0.4
37.5	6.25	7.2 ± 1.0	9.6 ± 0.4
50	0	1.3 ± 0.4	5.1 ± 0.3
75	0	1.1[g] ± 0.5	0.6[h] ± 0.3

[a] H_2O_2 remaining after addition of various concentrations of Fe^{2+} to 25 μM H_2O_2 in the absence or presence of oxygen. The order of addition of reagents was: phosphate buffer, pH 7.4; 25 μM H_2O_2; Fe^{2+}; then 500 U/mL catalase.

[b] The expected concentration of H_2O_2 remaining when assuming a stoichiometry of 2 Fe^{2+} for 1 H_2O_2 in the Fenton reaction, Reactions 17–19.

[c] Anaerobic solutions were achieved by bubbling with argon for at least 10 min.

[d] Air-saturated solution; thus initial $[O_2] \approx 250$ μM.

[e] Experimentally observed $[H_2O_2]$ after addition of catalase.

[f] Data are expressed as means ± SE from $n = 3$.

[g] Background in anaerobic condition.

[h] Background in aerobic condition.

Chelators can enhance Fe^{2+}-induced radical formation

Ethylenediaminetetraacetic acid and DTPA are widely used as iron chelators in biological research because they can drastically alter the efficiency of iron as a catalyst in oxidation reactions, e.g., the Haber-Weiss reaction. Both are able to enhance the rate of iron autoxidation as well as enhance radical yield in the Fenton

Table 2. Antioxidant Enzymes Decrease POBN Adduct Yield in Fe^{2+} + O_2 Oxidations

Treatment	DMSO oxidation [POBN adduct] (nM)[a]	EtOH oxidation [POBN adduct] (nM)	Glucose oxidation [POBN adduct] (nM)
Fe^2 alone[b]	200 ± 13.5[c]	290 ± 12.5	33 ± 2.0
Fe^2 and BSA[d]	190 ± 30	280 ± 23	31.7 ± 1.5
Fe^2 and 50 U/mL SOD	142 ± 16*	191 ± 33*	28.8 ± 1.8
Fe^2 and 500 U/mL CAT	171 ± 17	210 ± 18*	30.1 ± 1.7
Fe^2 and SOD + CAT[e]	100 ± 11†	120 ± 9†	21.6 ± 1.6*

[a]POBN adduct yield.
[b]Control group; Fe^{2+} is 100 μM in all treatments.
[c]Data are expressed as means ± SE from $n = 3$.
[d]BSA at the same protein concentration of the SOD + CAT experiment below.
[e]SOD + CAT: 50 U/mL SOD + 500 U/mL CAT.
*$p < .05$ compared with control.
†$p < .001$ compared with control.

reaction [15,31]. However, DTPA greatly reduces the rate of the iron-catalyzed Haber-Weiss reaction; EDTA can enhance the iron-catalyzed Haber-Weiss reaction [34]. Thus, the effect of DTPA and EDTA on Fe^{2+}-enhanced radical formation was studied by EPR spin trapping with POBN. All target-radical formation, as monitored by EPR spin trapping, was significantly enhanced by Fe^{2+}-DTPA and Fe^{2+}-EDTA compared with aqueous Fe^{2+} ($p < 0.001$), Fig. 3. In the oxidation of DMSO radical formation was enhanced 13-fold by EDTA and 8-fold with DTPA; for EtOH oxidation, 17-fold for EDTA and 7-fold for DTPA; in glucose oxidation, 14-fold with EDTA and 7-fold for DTPA. These enhancements are even greater than the enhancement observed via the Fenton reaction using 100 μM H_2O_2 and the same iron concentration. This supports our hypothesis that Fe^{2+}-dioxygen chemistry produces species that are important initiators of free radical oxidations, especially when appropriate metal chelators are available.

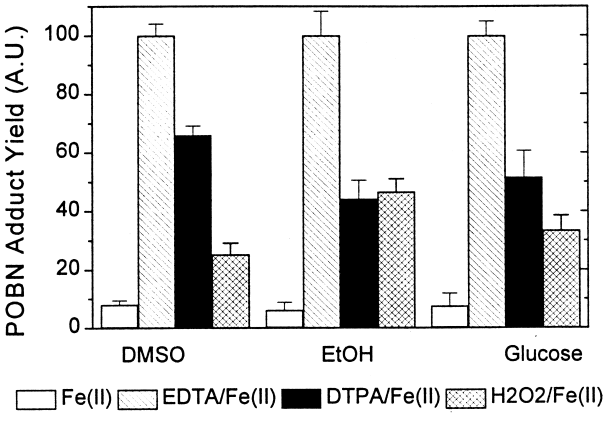

Fig. 3. EDTA and DTPA enhance target molecule oxidation by 'Fe^{2+} + O_2'. One hundred μM Fe^{2+}-dependent DMSO (100 mM), EtOH (100 mM), and glucose (200 mM) oxidations are greatly enhanced by these chelators.

The ratio of $[O_2]/[H_2O_2]$ affects the reaction mechanisms

Based on literature reports, the estimated physiological ratio of $[O_2]/[H_2O_2]$ would be about $10^3 \sim 10^5$. This high ratio in favor of dioxygen suggests from kinetic considerations that in a biological setting, the rate of substrate oxidation due to iron-oxygen complexes might be much higher than the oxidation-rate due to the Fenton reaction from pre-existing H_2O_2. To examine our proposal, we compared Fenton-mediated radical formation vs. 'Fe^{2+} + O_2'-mediated radical formation, using POBN adduct formation as a marker. From these data we can infer the dependence on $[O_2]/[H_2O_2]$ of radical formation by the Fenton reaction and by 'Fe^{2+} + O_2' chemistry, Fig. 4. All targets examined show that the relative importance of the Fenton reaction (as measured by substrate oxidation) significantly decreases as $[O_2]/[H_2O_2]$ increases. For example, when $[O_2]/[H_2O_2] <$ 10, radical yield from the Fenton reaction dominates, as seen when the ordinate of Figure 4 > 1.[1] However, when $[O_2]/[H_2O_2] \geq 100$, the radical yield from the Fenton reaction is minor (even negligible; Fig. 4 ordinate values approach 1) compared to that from a simple aerobic Fe^{2+} solution. Thus, when $[O_2] >> [H_2O_2]$, free radical oxidations initiated by 'Fe^{2+} + O_2' chemistry dominate. Furthermore, based on an estimated physiologic $[O_2]/[H_2O_2]$ $\geq 10^3$, we can easily infer that the Fenton reaction with pre-existing H_2O_2 is in general only a minor initiator; thus, we suggest that species arising from Fe^{2+}-dioxygen chemistry are significant initiators of free radical-mediated oxidations.

[1]The ordinate of Fig. 4 represents the ratio of EPR signal height of the POBN spin adduct produced in an aerobic system upon introduction of Fe^{2+} with H_2O_2 present compared to the signal height in an aerobic system without H_2O_2. An ordinate value of 5 indicates there is five times as much POBN spin adduct produced with H_2O_2 present compared to when no H_2O_2 was added. In the limit as $[H_2O_2]$ approaches zero, i.e., the abscissa ($[O_2]/[H_2O_2]$) approaches infinity, the curves will all approach an ordinate value ($S.H._{[H2O2 + O2)}/S.H._{(O2)}$) of one, i.e., the numerator and denominator of the ordinate are the same experiment.

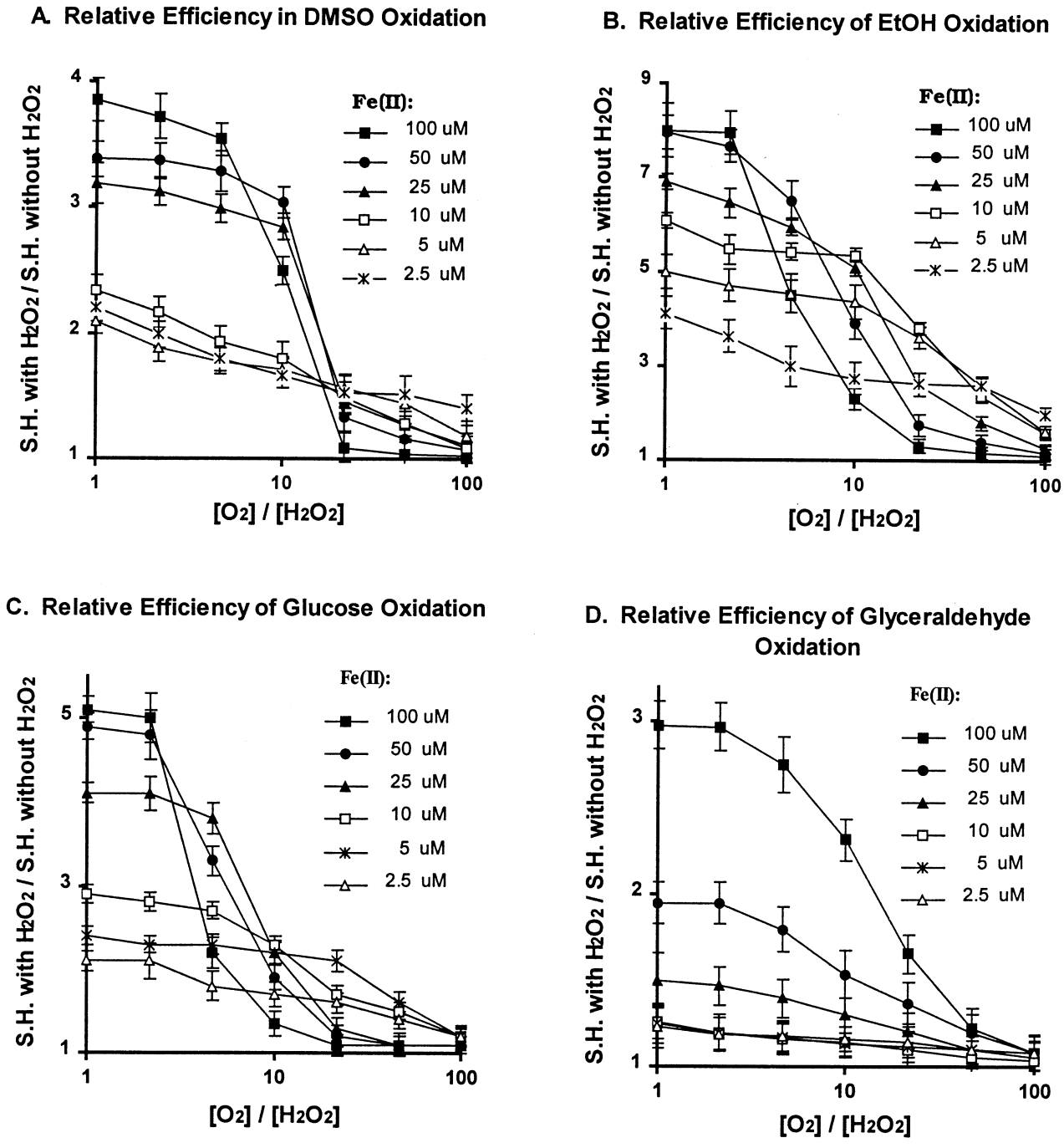

Fig. 4. 'Fe^{2+} + O_2' chemistry dominates initiation of radical production when $[O_2]/[H_2O_2] \geq 100$. The ordinate represents the ratio of EPR signal height (S.H.) of the POBN spin adduct in an aerobic system in the presence of H_2O_2 compared with the signal height in an aerobic system without H_2O_2. (A) 100 mM DMSO; (B) 100 mM EtOH; (C) 200 mM glucose; and (D) 100 mM glyceraldehyde were incubated with 2.5, 5.0, 10, 25, 50, 100 μM Fe^{2+} with/without 2.5, 5.0, 10, 25, 50, 100 and 250 μM H_2O_2 under aerobic conditions. All solutions were air saturated, thus the initial $[O_2]$ is about 250 μM. Values were expressed as means ± SD (n = 3).

Cellular free radical oxidations are not enhanced by H_2O_2

Iron-mediated cellular lipid peroxidation is thought to be one of the key factors in cell injury. Polyunsaturated fatty acyl groups in lipids located in cell membranes are the principal targets for cellular lipid peroxidation. To determine if 'Fe^{2+} + O_2' chemistry is more important than the Fenton reaction in initiating membrane lipid peroxidation in cells, we enriched the membranes of

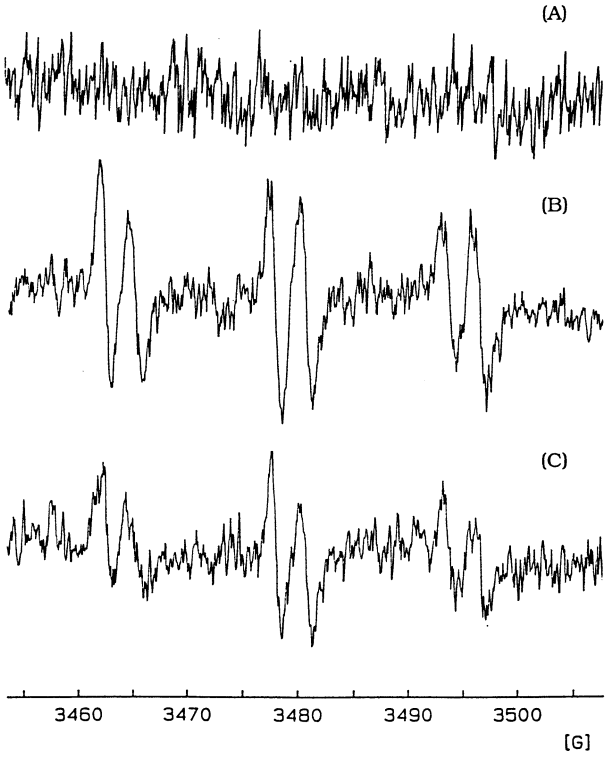

Fig. 5. 'Fe^{2+} + O_2' initiates lipid radical formation in cells. (A) L1210 + 50 mM POBN; (B) L1210 + 50 mM POBN + 100 μM Fe^{2+}; (C) L1210 + 50 mM POBN + 50 μM H_2O_2 + 100 μM Fe^{2+}; All solutions were air-saturated before addition of Fe^{2+}.

L1210 murine leukemia cells with docosahexaenoic acid (DHA, 22:6n-3). We then subjected them to either Fe^{2+} + O_2 or Fe^{2+} + H_2O_2 + O_2. Using POBN as the spin trap to detect carbon-centered radicals arising from cellular lipid peroxidation, we observed that Fe^{2+} and dioxygen results in substantial lipid-derived radical formation in the L1210 aerobic cell suspension. However, consistent with earlier observations on cellular injury using enzyme activity [35], the inclusion of H_2O_2 into the cell suspension before introduction of Fe^{2+} failed to produce additional POBN lipid-derived radical adduct formation. In fact the addition of H_2O_2 (25–100 μM) inhibits POBN adduct formation, Fig. 5, again suggesting that cellular oxidation is mediated predominately by Fe^{2+}-dioxygen chemistry. Our observation that Fe^{2+}-dioxygen chemistry is important in cellular lipid peroxidation is in agreement with work on models of lipid systems that have demonstrated the autoxidation of ferrous iron can initiate lipid peroxidation [10,36,37]. Superoxide dismutase and CAT can provide protection in some circumstances. The ability of ferrous iron to initiate lipid peroxidation and the ability of SOD and CAT to provide some protection is a function of the specific coordination environment of ferrous iron.

In summary, uncontrolled iron-catalyzed oxidations can easily damage DNA [38], proteins [39], and lipids [32,40]. Consequently, cells have developed efficient strategies to protect themselves from potential $HO^{\bullet}$ formation arising from Fe^{2+} and pre-existing H_2O_2. H_2O_2 is removed by the glutathione peroxidases or catalase and iron is carefully managed by organisms. The data presented here suggest that iron needs to be carefully controlled to protect not only against the Fenton reaction with pre-existing hydrogen peroxide, but perhaps more importantly to minimize oxidative damage initiated by Fe^{2+}-dioxygen chemistry. Please note that we are not concluding that the Fenton reaction is not to be considered in understanding damaging biological oxidations.

Our experiments with SOD and CAT clearly demonstrate that $O_2^{\bullet -}$ and H_2O_2 are involved in the oxidations initiated by Fe^{2+}-dioxygen chemistry. The Fenton reaction would be operative, but it explains only a small part of the observations. The major point we wish to make is that pre-existing H_2O_2 is not required for ferrous iron to be a detrimental species in biological systems. We propose that Fe^{2+} and dioxygen lead to oxidative species that must be considered as major players in deleterious free radical-mediated biological oxidations.

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ABBREVIATIONS

AU—arbitrary units
BSA—bovine serum albumin
CAT—catalase
DHA—docosahexaenoic acid
DTPA—diethylenetriaminepentaacetic acid
EDTA—ethylenediaminetetraacetic acid
EPR—electron paramagnetic resonance
Fe-O—iron-oxygen complexes
$Fe^{2+} + O_2$—species derived from iron(II) and dioxygen chemistry
POBN—α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone
S.H.—signal height
SOD—superoxide dismutase