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Original Contribution

ACIDIC pH AMPLIFIES IRON-MEDIATED LIPID PEROXIDATION IN CELLS

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Abstract—The goal of our study was to investigate the mechanism by which changes in extracellular pH influence lipid peroxidation processes. Ferrous iron can react with hydroperoxides, via a Fenton-type reaction, to initiate free radical chain processes. Iron is more soluble at lower pH values, therefore we hypothesized that decreasing the environmental pH would lead to increased iron-mediated lipid peroxidation. We used Photofrin, a photosensitizer that produces singlet oxygen, to introduce lipid hydroperoxides into leukemia cells (HL-60, K-562, and L1210). Singlet oxygen reacts with the PUFA of cells producing lipid hydroperoxides. Using EPR spin trapping with POBN, free radical formation from HL-60 cells was only detected when Photofrin, light, and ferrous iron were present. Free radical formation increased with increasing iron concentration; in the absence of extracellular iron, radical formation was below the limit of detection and lipid hydroperoxides accumulated in the membrane. In the presence of iron, lipid-derived radical formation in cells is pH dependent; the lower the extracellular pH (7.5–5.5), the higher the free radical flux; the lower the pH, the greater the membrane permeability induced in K-562 cells, as determined by trypan blue dye exclusion. These data demonstrate that lipid peroxidation processes, mediated by iron, are enhanced with decreasing extracellular pH. Thus, acidic pH not only releases iron from "safe" sites, but this iron will also be more damaging. © 2000 Elsevier Science Inc.

Keywords—Lipid peroxidation, Free radical, Oxidative stress, EPR, Photofrin, Singlet oxygen

INTRODUCTION

There are numerous pathologies that have associated oxidative stress as well as low tissue-pH values [1,2]. For example, the extracellular pH in human tumors can vary from 7.68–5.85 [1]. These lower pH environments could not only influence metabolic processes, but they may also affect the detrimental oxidative processes in the tissue. Iron is often a key player in oxidative stress [3–5]. In lipid peroxidation iron can both initiate and amplify free radical-mediated lipid peroxidation reactions [6–9]. The pH of tissue could modulate the ability of iron to participate in detrimental lipid peroxidation reactions. It has been observed that pH can affect the rate of lipid peroxidation [7,8,10] but mechanistic aspects were not always addressed. Hanlon and Seybert found that pH influenced the effectiveness of azo initiators to start lipid peroxidation [10]. Iron was not involved in these experiments. In both the work of Wills [7] and Barber [8] with tissue homogenates or microsomes, lipid peroxidation was accelerated as the pH changed from 8 to 5. However, the changing chemistry of iron as the pH is changed was not addressed. Both iron (II) and iron (III) are more soluble at lower pH, making them more available for oxidative processes. Thus, we hypothesized that as the extracellular pH is lowered, there will be a corresponding increase in the flux of lipid-derived free radicals induced by iron. To test this hypothesis we used singlet oxygen as a tool to generate lipid hydroperoxides. Singlet oxygen was generated in leukemia cell membranes by Photofrin, a photosensitizer used in the photodynamic therapy of cancer [11,12]. Singlet oxygen adds to the double bonds of unsaturated fatty acids producing LOOHs (Scheme 1) [13–15]. We used EPR spin trapping with POBN to detect lipid-derived radical formation from leukemia cells [16] as the pH was varied during the oxidative stress produced by singlet oxygen and iron; membrane permeability was determined with the trypan blue dye exclusion assay.

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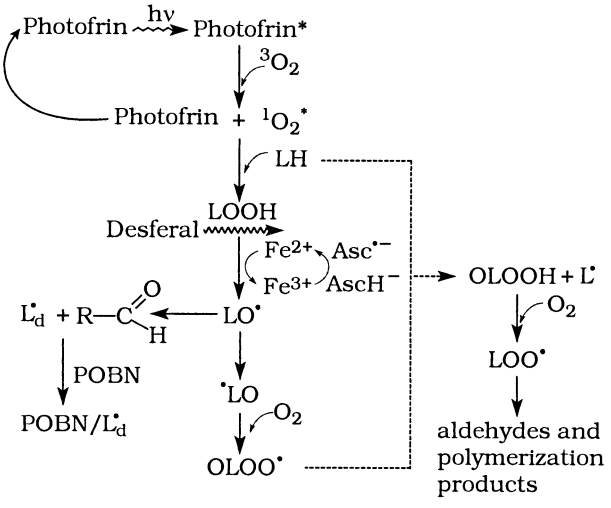
MATERIALS AND METHODS

Chemicals

Photofrin (porfimer sodium, QLT, Phototherapeutics Inc., Vancouver, BC, Canada) was dissolved in 5% dextrose (pH 7.4) sterile filtered and frozen at −20°C. α-(4-Pyridyl-1-oxide)-N-*tert*-butylnitrone (Aldrich Chemical Co., Milwaukee, WI, USA) was prepared as a 1.0 M stock solution in distilled water. Desferal (Ciba Pharmaceutical Co, Summit, NJ, USA) was dissolved in PBS pH 7.5 to a final concentration of 100 μM. PBS, 210 mg potassium dihydrogen phosphate, 407 mg sodium monohydrogen phosphate and 9 g sodium chloride were dissolved in 1 l of water and adjusted to pH 7.4 (or lower pH) with 1 N HCl. The PBS solution was sterile filtered and stored over chelating resin (Sigma Chemical Co., St. Louis, MO, USA) to minimize the level of adventitious transition metals [17]. Ascorbic acid (10 mM) and ferrous iron (1.0 mM $FeSO_4$) stock solutions in distilled water were freshly prepared before each experiment.

Cell culture

Leukemia cells were acquired from American Type Culture Collection (ATCC, Rockville, MD, USA). All cells were grown in medium consisting of RPMI 1640 medium (Gibco/Life Technologies, Grand Island, NY, USA) and 10% fetal bovine serum. HL-60 stock cells were grown in this medium supplemented with L-glutamine (2 mM). Experiments were done when cells were in exponential growth phase.



Scheme 1. Mechanism of how singlet oxygen can promote lipid peroxidation.

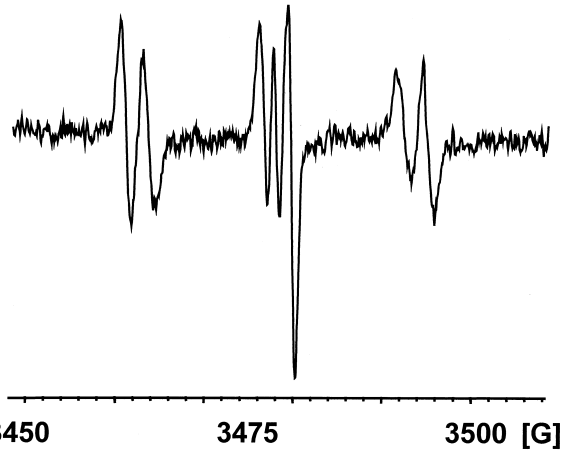


Fig. 1. EPR spectrum of a POBN lipid-derived radical adduct from leukemia cells. Typical EPR spectrum of the lipid-derived radical trapped by POBN, (a^N = 15.6 G; a^H = 2.6 G; also seen is the $Asc^{\bullet-}$ doublet, a^H = 1.8 G) generated from cells subjected to the oxidative stress of Photofrin, light, iron and ascorbate. Instrument settings were: Mod amp, 1.0 G; power, 20 mW; frequency 9.764 GHz; receiver gain, 2.0×10^5; time constant, 82 ms; sweep rate 60 G/42 s. This spectra is the result of 15 summed scans.

Photofrin uptake

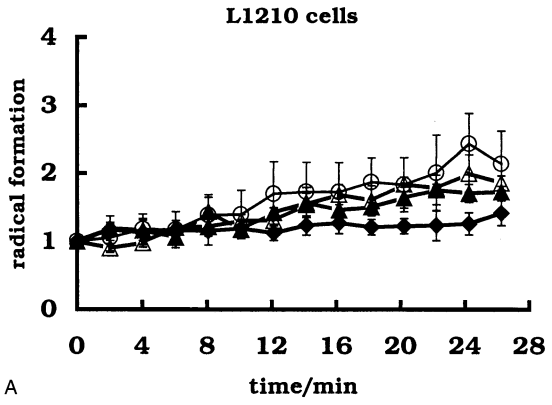
Cells were exposed to Photofrin (0.9 or 9 μg/ml) at 37°C for 45 min in PBS pH 7.4. Using always the same buffer pH for Photofrin uptake ensured that for each type of experiment, Photofrin was constant. After incubation with Photofrin, cells were centrifuged (300 g) and resuspended in PBS pH 7.5–5.5.

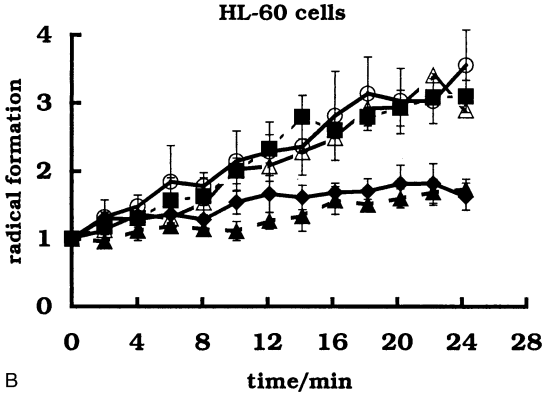
Membrane permeability

Trypan blue dye exclusion was used to determine membrane permeability. Cells (4×10^6/ml) were exposed to Photofrin (0.9 μg/ml) for 45 min, washed and resuspended in 1 ml PBS. Ascorbic acid (100 μM) and ferrous iron (5 μM $FeSO_4$) were added to 500 μl cell samples (2×10^6 cells) and placed into wells of a 48-well dish. Cells were then exposed to light on a light box (5 J/m^{2}s). Every 2 min a 20 μl sample was removed, mixed with 80 μl trypan blue solution (0.2%) and then cells were counted using a microscope. The light box used for this experiment (TRU-VIEW light box, Logan Electric, Spec. Mfg. Co. Chicago, IL, USA) was equipped with a circular Sylvania light bulb (daylight, 22W) covered with a light diffuser that is impermeable to UV. The intensity was measured with a Yellow Spring Instrument 65 A Radiometer with the 6551 probe.

EPR experiments

Cells (8×10^6/ml) were incubated with Photofrin (9 μg/ml) for 45 min, washed and resuspended in 1 ml PBS





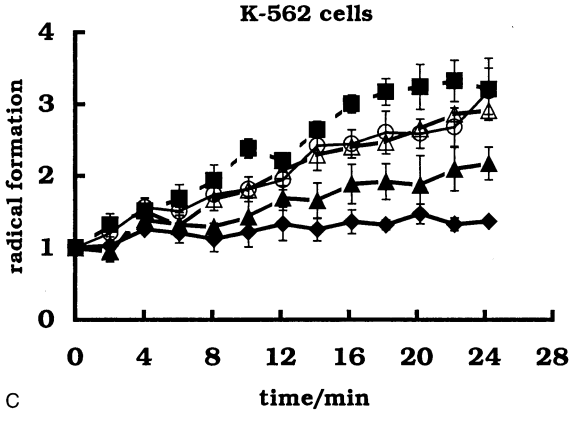
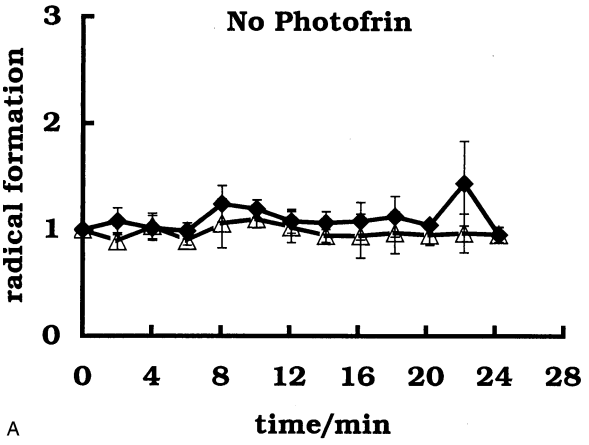


Fig. 2. Lipid radical formation in leukemia cells is pH dependent. EPR signal intensity of POBN/$L_d^{\cdot}$ produced from HL-60, K-562 and L1210 cells (8×10^6 cells/ml) is shown. Cells were treated with Photofrin (9 μg/ml) for 45 min, then washed and resuspended in PBS (pH 7.5–5.5) containing 100 μM ascorbate and 5 μM ferrous iron. For the first data point cells were kept in the dark, then for the following data points cells were continuously exposed to light (180 J/m^{2}s) in the EPR cavity. ■, pH = 5.5; △, pH = 6.0; ○, pH = 6.5; ▲, pH = 7.0; ◆, pH = 7.5. (A) L-1210 cells; (B) HL-60 cells; (C) K-562 cells. To allow easy comparison between experiments, EPR signal intensity was normalized to that seen in the dark; i.e., the first data point of each experiment. At the first data point the POBN/$L_d^{\cdot}$ spin adduct was below the limit of detection. Thus, "1" represents the instrument noise level. Experiments were done in triplicate, standard errors are shown.



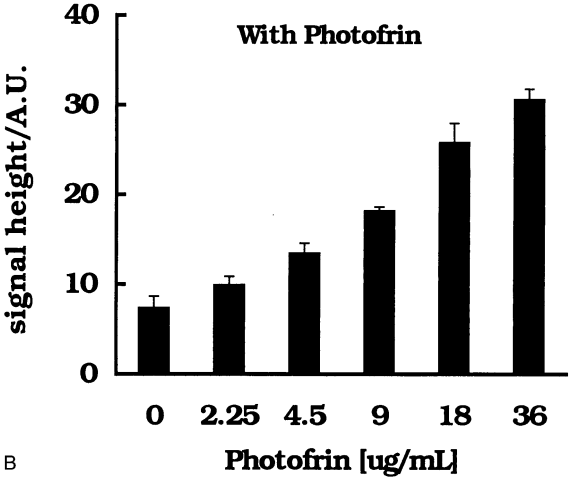


Fig. 3. Without light or iron no detectable radical formation from HL-60 cells was observed. (A) EPR signal intensity of POBN/$L_d^{\cdot}$ produced from HL-60 cells (8×10^6 cells/ml) suspended in PBS pH 7.5 or 6.5 with 5 μM ferrous iron and 100 μM ascorbate is shown. At both pH 7.5 (◆) and 6.5 (△) radical formation is below the limit of detection by POBN spin trapping when HL-60 cells were subjected to visible light (tungsten, 180 J/m^{2}s) in the absence of Photofrin. (B) Free radical formation is dependent on the amount of Photofrin. EPR signal intensity of POBN/$L_d^{\cdot}$ produced from L1210 cells (8×10^6 cells/ml). Cells were treated with various amounts of Photofrin (0-36 μg/ml) for 45 min in PBS pH 7.5. Cells were then resuspended in 0.9% NaCl with POBN (25 mM) and ascorbate (100 μM) and illuminated with light (tungsten, 180 J/m^{2}s) for 2 min. Ferrous iron (5 μM) was added to the sample and radical formation measured by EPR.

(pH 7.5–5.5). An aliquot of 500 μl was mixed with POBN (25 mM), ascorbate (100 μM), and $FeSO_4$ (5 μM) and placed into a TM_{110} EPR quartz flat cell. Ferrous iron and ascorbic acid were needed to initiate radical formation from lipid hydroperoxides. Cells were exposed to visible light (tungsten, 180 J/m^{2}s) directly in the EPR cavity and the POBN radical adduct EPR signal intensity

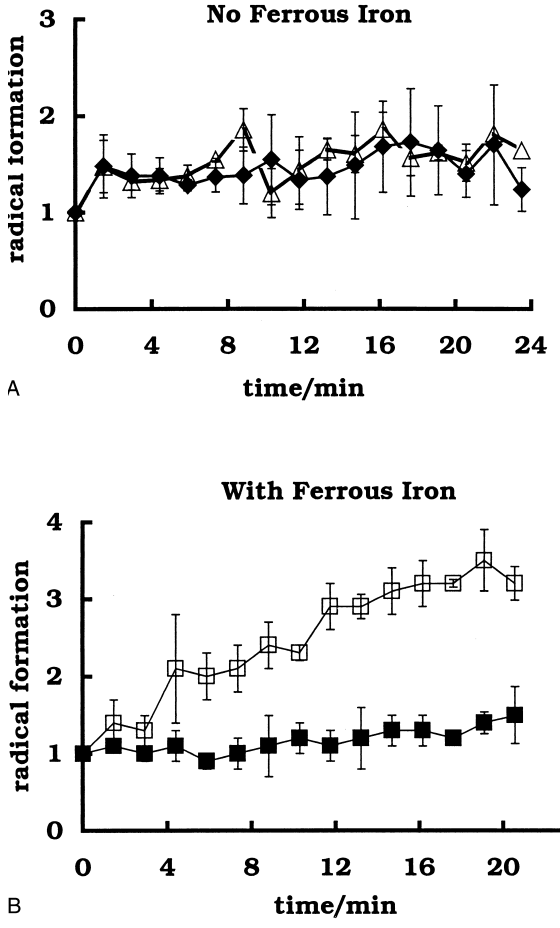


Fig. 4. Photofrin and light induce radical formation in HL-60 and K-562 cells only when extracellular ferrous iron is present. EPR signal intensity of POBN/$L_d^{\bullet}$ produced from HL-60 cells (8×10^6 cells/ml) is shown. Cells were treated with Photofrin (9 μg/ml) and light (tungsten, 180 J/m^{2}s) and a combination of: Fe^{2+} (5 μM) and ascorbate (100 μM) in PBS pH 7.5 or 6.5. (A) No ferrous iron: HL-60 cells in PBS pH 7.5 (◆) and 6.5 (△) with ascorbate. (B) With ferrous iron: HL-60 in PBS pH 7.5 (■) and 6.5 (□) with ascorbate and 5 μM ferrous iron.

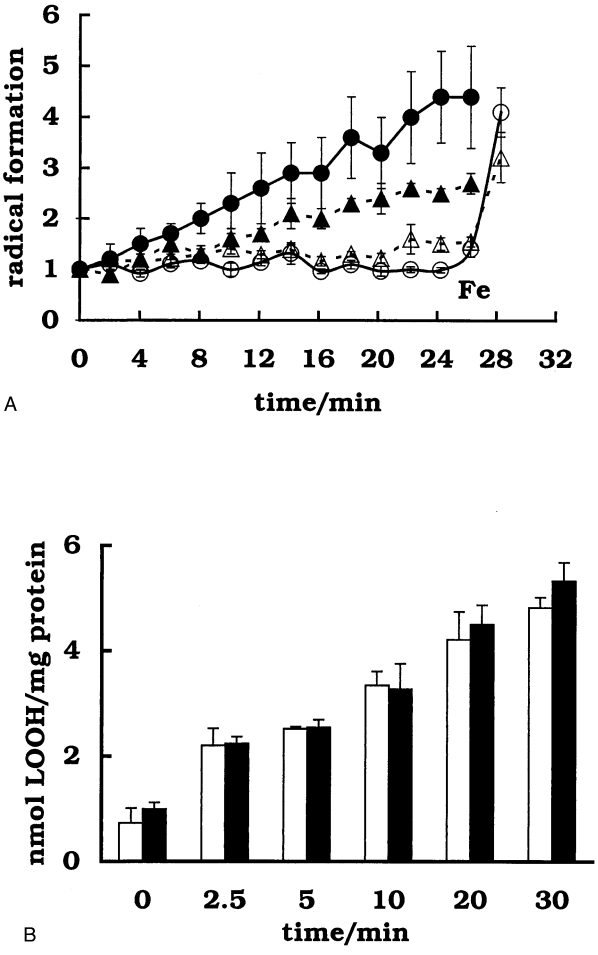


Fig. 5. Without Fe^{2+}, LOOH accumulates. (A) HL-60 and K-562 cells were incubated with/without 100 μM Desferal (during Photofrin uptake) and then exposed to light in PBS (pH 6.5). Ferrous iron (5 μM) was added to HL-60 cells (●) and K-562 cells (▲) in PBS pH 6.5 at the beginning of the experiment; Desferal was not present. HL-60 cells (○) and K-562 cells (△) were incubated with Desferal during Photofrin uptake. Ferrous iron (50 μM) was added after 26 min of light exposure to the cells in PBS pH 6.5 containing 100 μM Desferal. (B) HL-60 cells were incubated with 9 μg Photofrin and then exposed to light in PBS 7.5 or 6.5. LOOH were determined using Fe-thiocyanate as a chromogen at 500 nm. White columns represent pH 6.5, black columns pH 7.5.

was monitored versus time. Each data point represents the signal-averaged result of 5 scans of the low field doublet of the POBN/lipid-derived radical adduct spectrum (Fig. 1). The first five scans were performed in the dark. In Figs. 2–6, the first data point representing the POBN/$L_d^{\bullet}$ spin adduct concentration was below the limit of detection for the instrument parameters used. Thus, "1" represents the instrument noise level. EPR experiments were performed with a Bruker ESR-300 EPR spectrometer (Karlsruhe, Germany).

Desferal experiments

Cells were exposed to Photofrin in 100 μM Desferal-PBS pH 7.5 for 45 min at 37°C. After centrifugation (300 g) cells were resuspended in 100 μM Desferal-PBS and used for EPR or trypan blue experiments.

LOOH assay

Determination of total lipid hydroperoxides were done with the Cayman LPO Assay Kit (Cat# 705002). In this kit the unstable hydroperoxides react with ferrous ions; the resulting ferric ions are detected using thiocyanate ion as the chromogen. HL-60 cells (6×10^6 cells/ml) were exposed to Photofrin (9 μg Photofrin/ml) in PBS pH 7.5. Cells were washed and resuspended in PBS pH 6.5 or 7.5 and transferred to a 48 well plate (6×10^6 cells/well). Cells

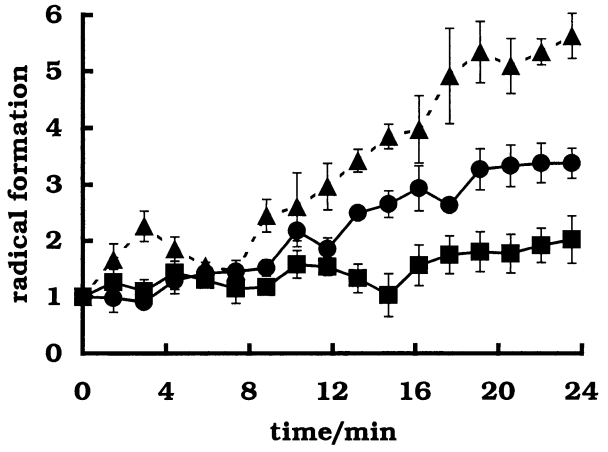


Fig. 6. The amount of extracellular ferrous iron changes radical yield from HL-60 cells exposed to singlet oxygen, as seen by the EPR signal intensity of POBN/$L_d^{\bullet}$. Cells (8×10^6 cells/ml) were treated with Photofrin (9 μg/ml), washed and resuspended in PBS containing 100 μM ascorbate and various amount of ferrous iron (0, 5, 25 μM). Symbols: ■, 0 μM ferrous iron; ●, 5 μM ferrous iron; and ▲, 25 μM ferrous iron.

were then exposed to light (5 J/m^{2}s) for 0–30 min. After light exposure cells were transferred to 1 ml Eppendorf tubes, centrifuged (300 g, 4 min) and resuspended in 500 μl PBS pH 7.5. Samples were kept on ice during the following treatment. Cells were homogenized with a Vibra Cell sonicator (Sonics & Materials Inc. Danbury, CT, USA) for 20 s (output 10, 40% Duty cycle). Samples (450 μl) were transferred into 15 ml tubes and extracted with extract R and chloroform, following the protocol of the LPO Assay Kit. Standard curve and samples were read at 500 nm with a spectrometer (HP Kayak XA).

Statistics

Experiments were done in triplicate; standard errors are shown.

RESULTS AND DISCUSSION

Free radical formation induced by singlet oxygen is pH dependent

Singlet oxygen can react with double bonds of unsaturated fatty acids to produce lipid hydroperoxides (Scheme 1). In the presence of ferrous iron, these lipid hydroperoxides can undergo free radical chain reactions, resulting in formation of oxygen- and carbon-centered lipid radicals. The carbon-centered, lipid-derived radicals can be detected by EPR using POBN as a spin trap (Fig. 1). Because iron is more soluble at acidic pH values, and thus is more available for redox reactions, we hypothesize that decreasing extracellular pH will lead to increased cellular lipid peroxidation. To test this, we used Photofrin, a photosensitizer used in cancer therapy that locates in cell membranes as a source of singlet oxygen for the introduction of LOOH [11,12]. We exposed HL-60, K-562 and L1210 cells to Photofrin, light, 100 μM ascorbate ($AscH^-$) and 5 μM Fe^{2+} and detected the resulting carbon-centered lipid-derived radicals with EPR spin trapping [16,18–20]. Ascorbate was included to recycle Fe^{3+} back to Fe^{2+} (Scheme 1). As seen in Fig. 2, free radical formation is pH dependent for all three cell lines. While radical formation from L1210 cells is only slightly increased with decreasing pH, radical formation in HL-60 cells increases significantly at pH values less than 7.0. K-562 cells are even more sensitive to extracellular pH changes; free radical formation continuously increases when the pH is lowered from 7.5–5.5.

Because several factors (Photofrin, light, ferrous iron, pH) are involved in the formation of lipid-derived radicals seen in these cells, we examined what role each factor plays in this pH-dependent radical formation. Experiments were designed where one or more of the factors were missing.

No radical formation was detectable when HL-60 cells were exposed to light and extracellular iron

Leukemia cells were used to determine the role of extracellular iron and pH on singlet oxygen-induced lipid peroxidation. To test if light and iron without a singlet oxygen source can induce radical formation, HL-60 cells were exposed to light (180 J/m^{2}s) in the presence of 5 μM $FeSO_4$, and EPR spectra were collected. Although light can induce $HO^{\bullet}$ formation from iron chelates [21], under our experimental conditions, light and iron do not induce detectable radical formation in HL-60 cells (Fig. 3A). Changes in extracellular pH did not change the outcome of this experiment. Thus, without Photofrin, free radical formation is below the limit of detection when cells are exposed to Fe^{2+} (5 μM) and light.

The presence of iron results in detectable radical formation

Singlet oxygen will react with unsaturated membrane lipids producing lipid hydroperoxides in a nonradical mechanism [13,14]. Ferrous iron can react with these hydroperoxides initiating free radical chain reactions. This radical formation is dependent on the amount of Photofrin (Fig. 3B). The experiments in Fig. 4 demonstrate that iron is required for radical formation. In the absence of iron at both pH 7.5 and 6.5, no lipid-derived radicals were detected from HL-60 cells subjected to a flux of singlet oxygen. In the presence of 5 μM Fe^{2+}, radical formation was detected but only at acidic pH (6.5). These data suggest that acidic pH allows iron to be more available to participate in the chain reactions of lipid peroxidation.

In the absence of iron, lipid hydroperoxides accumulate in cell membranes

If iron is required for the initiation of free radical chain reactions from lipid hydroperoxides, then in the absence of iron, LOOH should accumulate in cell membranes exposed to a flux of singlet oxygen (Scheme 1). To ensure that adventitious redox active transition metals, including iron, are not available for reaction with LOOH, we included the chelator Desferal in the cell incubations. Desferal strongly chelates ferric iron (pK_s = 30.6) while weakly chelating ferrous iron (pK_s = 7.2) [22–24]. Thus, Desferal stops (or greatly slows) the redox cycling of iron. However, the introduction of ferrous iron to an aerated Desferal-containing cell system will result in the production of free radicals because of the Desferal-driven conversion of ferrous to ferric iron. This is a common event with chelating agents that prefer ferric over ferrous iron [25,26]. Thus, when ferrous iron is added to a system that contains peroxides and Desferal, free radical reactions will be initiated. To determine if lipid hydroperoxides accumulate in cells in the absence of ferrous iron, EPR spectra were gathered while HL-60 and K-562 cells were exposed to Photofrin and light in the presence of Desferal (Fig. 5A). After 26 min of light exposure, radical formation was below the limit of detection. When ferrous iron was added to the cells a burst of radical production was observed (Fig. 5A). This burst of radical production upon the introduction of Fe^{2+} is consistent with the accumulation of LOOH. The radical formation from the Desferal treated cells detected after addition of Fe^{2+} is comparable to the flux of radicals of the control cells exposed to iron during the entire 26 min of light exposure. If adventitious iron is kept low, LOOH can accumulate without Desferal, as shown in Fig. 3B. These data demonstrate that lipid hydroperoxides produced by singlet oxygen can accumulate in the membrane and that ferrous iron is needed to initiate lipid-derived radical formation.

As further evidence for the accumulation of lipid hydroperoxides in the absence of catalytic metal, we measured lipid hydroperoxide formation with the LPO Assay. HL-60 cells were incubated with Photofrin and exposed to light in PBS that was pretreated with chelating resin to reduce the level of adventitious catalytic metals. Figure 5B shows that lipid hydroperoxides accumulate in a time dependent manner. Most importantly, these data demonstrate that the quantum yield of LOOH at the two pH values are the same. Thus, differences in radical formation are not the result of pH-dependent changes in the photodynamic properties of our sensitizer.

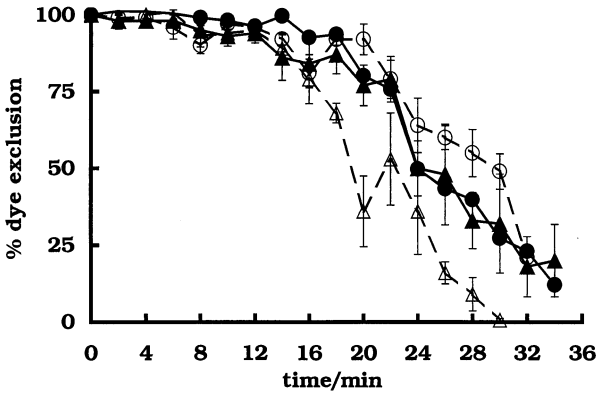


Fig. 7. Membrane damage induced by iron-mediated lipid peroxidation is enhanced at low pH. Trypan blue dye exclusion of K-562 cells (4×10^6 cells/ml) is shown. Cells were treated with Photofrin (0.9 μg/ml) for 45 min, washed and resuspended in PBS, pH 7.5 or 6.5, containing. Fe^{2+} (0 or 5 μM) and 100 μM ascorbate and then illuminated with visible light (5 J/m^{2}s). ●, pH 6.5 no Fe^{2+} added; ▲, pH 7.5 no Fe^{2+} added; ○, pH 7.5 with 5 μM Fe^{2+}; △, pH 6.5 with 5 μM Fe^{2+}.

Radical formation induced by singlet oxygen changes with amount of extracellular ferrous iron

To test the influence of different iron concentrations on lipid-derived radical formation, K-562 cells were exposed to 0, 5, and 25 μM ferrous iron during light exposure. As seen in Fig. 6, radical formation increases with increasing extracellular iron.

Low pH increases singlet oxygen-induced membrane permeability when extracellular iron is present

Singlet oxygen produces lipid hydroperoxides, which can compromise the integrity of the plasma membrane. The lipid hydroperoxides can undergo free radical chain reactions initiated by ferrous iron, amplifying the membrane damage, leading to cell death [18]. To examine this, membrane permeability of K-562 cells exposed to Photofrin, light and extracellular iron was determined using trypan blue dye. When the plasma membrane is damaged, trypan blue can no longer be excluded from the cell. At pH 7.5 membrane permeability of K-562 cells exposed to Photofrin and light is independent of extracellular iron; at pH 6.5 the membrane damage increased by a factor of 1.4 (change in time for 50% dye exclusion), when extracellular iron is present (Fig. 7). These data show that low pH enhances the membrane damage brought about by the presence of iron.

CONCLUSIONS

We have demonstrated that ferrous iron can amplify singlet oxygen damage in cells. In the absence of iron, LOOHs will accumulate in cell membranes. This oxidative damage results in loss of plasma membrane integrity, which

can lead to cell death. Ferrous iron amplifies oxidative damage to lipids by initiating free radical chain reactions. Acidic pH enhances the detrimental effect of iron. It is known that acidic pH in combination with reductants such as ascorbate can release iron from proteins like ferritin [7] and lactoferrin [27]. In our study we have addressed the catalytic efficiency of iron as the environmental pH changes. We have demonstrated that as pH decreases from 7.5 to 5.5, iron enhances cellular lipid peroxidation. Significant enhancement of radical production can be achieved with small pH changes, *e.g.*, changes from pH 7.5 to 7.0 (Fig. 2C). Thus, acidic pH not only releases iron from "safe" sites, but this iron will also be more damaging. Therefore, pH and iron need to be well controlled in cell experiments involving free radical oxidations. Our observations will be of importance in our understanding of pathology and treatment of disease. For example, the acidic environment associated with inflammation will exacerbate the oxidative damage mediated by iron. In cancer treatment the low pH of tumors could be used to advantage in therapies that have iron-catalyzed oxidations as a mechanism of action.

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ABBREVIATIONS

$AscH^-$—ascorbate monoanion
$Asc^{\bullet -}$—ascorbate radical
EPR—electron paramagnetic resonance
$L_d^{\bullet}$—lipid-derived carbon-centered radical
LOOH—lipid hydroperoxide
$^1O_2$—singlet oxygen
PBS—phosphate-buffer saline
PDT—photodynamic therapy
Photofrin—porfimer sodium
POBN—α-(4-pyridyl-1-oxide)-N-*tert*-butylnitrone
PUFA—polyunsaturated fatty acids