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Production of Lipid-Derived Free Radicals in L1210 Murine Leukemia Cells Is an Early Oxidative Event in the Photodynamic Action of Photofrin®

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ABSTRACT

Photofrin® photosensitization initiates a sequence of oxidative events that begins with singlet oxygen formation and ultimately leads to cell death. We hypothesize that membrane lipid-derived free radical formation is an early event in this process. In the presence of iron and ascorbate, lipid free radicals are generated during cellular photosensitization of L1210 cells as detected by electron paramagnetic resonance spin-trapping techniques. Tocopherol levels decline in an inverse manner to lipid radical formation. Trypan blue dye exclusion by membranes also decreases inversely to lipid radical formation but at an initially slower rate than α-tocopherol depletion. Propidium iodide nuclear staining as an alternative measure of cell integrity was a later event, occurring when α-tocopherol levels had fallen by 90%, trypan blue survival had decreased to below 10%, and lipid radical formation was nearing plateau levels. Likewise, the formation of cellular debris did not occur substantially until α-tocopherol was virtually exhausted and radical intensity had nearly reached a maximum. These temporal observations suggest the following sequence of events that leads to Photofrin® photosensitization-induced cytotoxicity in the presence of iron and ascorbate: (1) singlet oxygen-derived lipid hydroperoxide formation and subsequent radical production; (2) cellular α-tocopherol depletion; (3) trypan blue-detectable membrane leakage; (4) nuclear exposure to propidium; (5) cell disintegration. These observations are consistent with membrane lipid-derived free radical formation being an early and perhaps seminal event in photosensitization by Photofrin®, which leads to a concatenated series of events terminating in cell destruction.

INTRODUCTION

Photofrin®, a partially purified preparation of hematoporphyrin derivative, is being studied for use as a photosensitizer in the photodynamic therapy (PDT)† of cancer (1–3). Photodynamic action relies on the absorption of visible light by the photosensitizer to form its excited triplet state. This excitation energy is transferred to oxygen, producing singlet oxygen, $^1O_2$. This highly reactive, electrophilic oxygen species initiates oxidation processes that kill cells (1–3). The hydrophobic character of Photofrin® causes it to localize in plasma and subcellular membranes (4). Singlet oxygen, formed upon the illumination of Photofrin®, has been shown to initiate lipid peroxidation by an addition reaction to membrane polyunsaturated fatty acids (PUFA), forming lipid hydroperoxides in cells (4–9). It is known that L1210 cells and human CaSki cervical carcinoma cells that have a compromised ability to metabolize lipid hydroperoxides are significantly more sensitive to photosensitizer-produced singlet oxygen (4,7,10). We have previously shown that PDT with Photofrin® produces membrane-derived lipid free radicals and that increasing the polyunsaturation of cellular lipids enhances radical production as well as the resultant cytotoxicity (11). We have also shown that the presence of the prooxidants ferrous iron and ascorbate increases the flux of lipid radicals (11).

The above observations are consistent with the following molecular events.

$$L\text{–}H\ (PUFA) + {}^1O_2 \rightarrow LOOH \quad (1)$$

$$Fe^{2+} + LOOH \rightarrow LO^{\cdot} + OH^- + Fe^{3+} \quad (2)$$

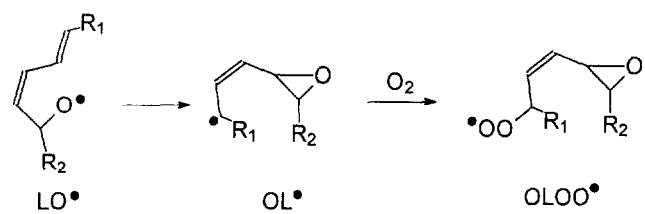
$$LO^{\cdot} \rightarrow L'C{=}O + {}^{\cdot}CH_2CH_3\ (L_d^{\cdot}) \quad (3a)$$

or

$$LO^{\cdot} \rightarrow OL^{\cdot} \quad (3b)‡$$

†*Abbreviations:* EPR, electron paramagnetic resonance; L', a carbon-centered radical on the intact lipid chain; $L_d^{\cdot}$, a carbon-centered radical on a fragment generated from the chain; LOOH, LOO' and LO', lipid hydroperoxide, lipid peroxyl and lipid alkoxyl radicals, respectively; PDT, photodynamic therapy; PI, propidium iodide; POBN, α-(4-pyridyl-1-oxide)-*N*-butylnitrone; POBN/$L_d^{\cdot}$, lipid-derived radical adduct of POBN; PUFA, polyunsaturated fatty acid.

‡Reactions 3b and 4 of LO', OL' and OLOO' can be represented structurally as:



The importance of OL' and subsequent formation of OLOO' has recently been demonstrated by Wilcox *et al.* (12).

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$$OL^{\cdot} + O_2 \rightarrow OLOO^{\cdot} \quad (4)$$

$$OLOO^{\cdot} + L\text{–}H \rightarrow L^{\cdot} + OLOOH \quad (5)$$

$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot} \quad (6)$$

$$LOO^{\cdot} + L\text{–}H \rightarrow LOOH + L^{\cdot} \quad (7)$$

$$POBN + L_d^{\cdot} \rightarrow POBN/L_d^{\cdot} \quad (8)$$

Although each of the radicals generated in the above scheme can in principle be trapped by α-(4-pyridyl-1-oxide)-*N*-butylnitrone (POBN), the principal spin-trapped species observed is the lipid-derived radical adduct of POBN (POBN/$L_d^{\cdot}$) because of the favorable trapping rate constant and the very long lifetime of this spin adduct (13,14).

Iron initiates this radical cascade by the reductive cleavage of LOOH (reaction 2). Ascorbate enhances these radical processes by reducing the Fe^{3+} formed in reaction 2 back to Fe^{2+}. Tocopherol can slow this process by quenching singlet oxygen (reactions 9 and 10) or by repairing the peroxyl radicals formed (reaction 11), thereby stopping the radical chain initiation reactions (reactions 5 and 7).

$$^1O_2 + TOH \xrightarrow{k_{pq}} O_2 + TOH \quad (9)$$

$$^1O_2 + TOH \xrightarrow{k_{cq}} \text{oxidized TOH} \quad (10)$$

and

$$OLOO^{\cdot} \text{ or}$$

$$LOO^{\cdot} + TOH \rightarrow OLOOH \text{ or } LOOH + TO^{\cdot} \quad (11)$$

where k_{pq} and k_{cq} are the physical and chemical quenching rate constants, respectively. Reactions 10 and 11 will consume TOH, thereby bringing about a loss of protection of the cell from these oxidative processes.

Because PDT with Photofrin® initiates lipid peroxidation in cells, it is important to know if lipid peroxidation and associated lipid radical formation happen before, during or after cell death. This information would help to delineate the role that lipid peroxidation and free radical processes play in the cytotoxicity of PDT. We report here a temporal study of lipid radical formation, α-tocopherol levels and several measures of cell integrity during Photofrin® photosensitization of cells.

MATERIALS AND METHODS

Photofrin® (porfimer sodium) was kindly provided by QLT Phototherapeutics, Inc., Vancouver, British Columbia, Canada. It was suspended in 5% dextrose/H_2O (pH 7.4) and frozen until immediately before use. Ascorbate was prepared as a 0.10 *M* stock solution of L-ascorbic acid (Aldrich Chemical Co., Milwaukee, WI) in distilled water. Concentration was verified by dilution in metal-free phosphate buffer, pH 7.4, using ϵ_{265} = 14 500 M^{-1} cm^{-1} for ascorbate (15). The POBN (OMRF Spin Trap Source, Oklahoma City, OK) was prepared as a 1.0 *M* stock solution in deionized H_2O. The concentration of Fe^{2+} was verified with Ferrozine, ϵ_{562} = 27 900 M^{-1} cm^{-1} (16).

The L1210 murine leukemia cells were grown in suspension culture at 37°C in medium consisting of RPMI-1640 (Grand Island Biochemical Co., Grand Island, NY) and heat-inactivated 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO). Cells were harvested during exponential growth phase and suspended in 0.9% NaCl for all experimental manipulations.

α-Tocopherol determinations were made using reverse-phase HPLC as previously reported (17). Briefly, L1210 cells were harvested by centrifugation and resuspended in ice-cold phosphate-buffered saline (17), pH 7.4. The α-tocopherol was extracted using ethanol and hexane and the extract was analyzed by injecting the samples onto a C-18 column with hexane/methanol 99:1 vol/vol as the mobile phase. Detection was accomplished by fluorescence with excitation at 292 nm and emission at 320 nm.

The PDT with Photofrin® was performed as follows: L1210 cells (8×10^6 cells/mL) were incubated in 0.9% NaCl with Photofrin® (15 μg/mL) in the dark for 45 min at 37°C. After this incubation, the cells were washed and suspended in fresh normal saline. To the suspension were added 100 μ*M* ascorbate and 5 μ*M* Fe^{2+}, in that order, and then the cells were exposed to visible light. For light treatment, a 2 mL aliquot of the cell suspension (8×10^6 cells/mL) was placed into a 12 × 75 mm borosilicate glass culture tube and illuminated for designated time points with a Bausch & Lomb 120 V 15 W Reflector Illuminator (tungsten bulb). The light was focused through a 5 cm layer of distilled water to remove the IR radiation; light intensity at the sample was 160 J m^{-2} s^{-1} as measured by a Yellow Springs Instrument 65A radiometer with the 6551 probe.

For the electron paramagnetic resonance (EPR) spin-trapping experiments, the Photofrin® incubation and reagent addition were performed as above except the sample size was 0.5 mL and the POBN was added prior to the Fe^{2+} ascorbate addition. Immediately after reagent addition, the cell suspension was transferred to a TM EPR quartz flat cell, which was then centered in a TM_{110} cavity of a Bruker ESP-300 EPR spectrometer and scans were initiated. Light intensity on the sample was 160 J m^{-2} s^{-1}. For the experiments in which the POBN radical adduct concentration was measured *versus* time, each data point represents the signal average of 10 scans of the low field doublet of the POBN/$L_d^{\cdot}$ spectrum. For these points, instrument settings were: 10 G/21 s scan rate; 1.0 G modulation amplitude; 1.0×10^6 receiver gain; 0.33 s time constant; and 40 mW nominal power (11).

The flow cytometry experiments were carried out using a Coulter EPICS 750 flow cytometer. Propidium iodide (PI) nuclear uptake was determined by exciting at 488 nm at 100 mW and detecting emission with a 635 nm bandpass filter. Cell debris formation was determined using forward angle light scatter and orthogonal light scatter. Data were analyzed with Coulter Elite (version 4.0) software.

RESULTS AND DISCUSSION

Lipid-derived radicals are produced in cells by Photofrin®

Lipid peroxidation results in the production of lipid peroxyl and perhaps alkoxyl radicals that react with the *bis*-allylic hydrogen atoms of adjacent PUFA, thereby propagating free radical chain reactions. We have developed an EPR spin-trapping technique using the spin trap POBN that allows the real-time detection of membrane-derived lipid free radical product from intact cells (11,18,19). When L1210 cells are incubated with Photofrin® and then exposed to light, we are able to observe lipid free radical production, as POBN/$L_d^{\cdot}$ (Fig. 1). The POBN/$L_d^{\cdot}$ adduct rises rapidly and begins to plateau at approximately 15 min of illumination under our experimental conditions. Cells that were incubated in the dark displayed no increase in POBN/$L_d^{\cdot}$ concentration in the same time frame. The hyperfine splittings of this POBN spin adduct (a^N = 15.65 G, a^H = 2.71 G) are consistent with the spin trapping of a lipid-derived free radical from the photodynamic action of Photofrin® on L1210 cells (11,18,19).

α-Tocopherol is consumed during Photofrin® photosensitization of cells

Photofrin® photosensitization produces singlet oxygen, and both singlet oxygen and the radicals formed during lipid peroxidation can deplete cell antioxidants. Therefore, we hy-

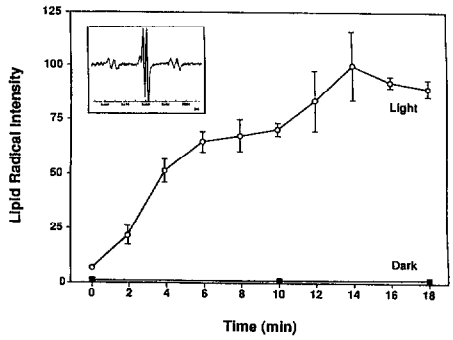

Figure 1. Lipid radicals are formed during Photofrin® photosensitization of L1210 cells. The EPR signal intensity of the POBN/L_d· produced from L1210 cells (8 × 10^6 cells/mL) with Photofrin® (15 μg/mL), Fe^{2+} (5 μ*M*), ascorbate (100 μ*M*) and POBN (25 m*M*). The light was turned on at the 0 time point and each data point represents the signal addition of the previous 2 min. (Insct) The EPR spectrum showing two radical species present, POBN/L_d· (a^N = 15.65 G, a^H = 2.71 G) and the ascorbate radical doublet (a^H = 1.8 G). The EPR instrument settings were 1.0 G modulation amplitude, 1 × 10^6 receiver gain; 40 mW nominal power and 60 G/336 s scan rate. The results represent the mean of at least three independent determinations.

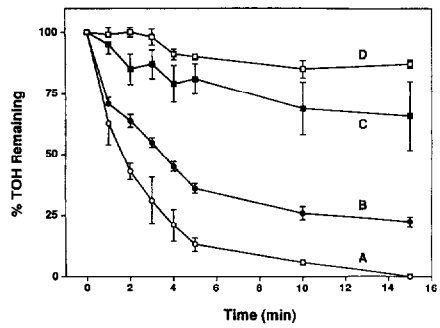

Figure 2. The photodynamic action Photofrin® depletes cellular α-tocopherol. The L1210 cells, incubated with Photofrin® (15 μg/mL), were illuminated in suspension (8 × 10^6 cells/mL) in 16 × 125 mm glass screw-cap tubes in the presence of Fe^{2+} (5 μ*M*) and ascorbate (100 μ*M*) and at the same light intensity as used in the EPR experiments. Tocopherol extractions were performed at designated time points and the data represent the means and SEM of at least three independent determinations. Shown are experiments with or without light and with or without iron/ascorbate. **A**) Photofrin®, light and Fe^{2+}/ascorbate; **B**) Photofrin®, light; **C**) Photofrin®, Fe^{2+}/ascorbate; and **D**) Photofrin® alone. The α-tocopherol level in these L1210 cell preparations prior to light exposure was 2.2 ± 0.17 μg/10^8 cells, which is identical to our previous observations (17,22).

pothesized that α-tocopherol levels in the cells should decrease during photosensitization. Indeed, upon photosensitization of the L1210 cells, there is a rapid decline of cellular α-tocopherol levels (Fig. 2). Under our experimental conditions, at 6 min, the time at which lipid radical production begins to slow, α-tocopherol levels have dropped by approximately 90%. The α-tocopherol level of cells that were not exposed to light declines modestly, probably due to the presence of the prooxidants Fe^{2+} and ascorbate. This loss of α-tocopherol during photosensitization mirrors the increase in POBN/L_d·. We are unable to distinguish definitely between singlet oxygen-mediated tocopherol depletion and tocopherol depletion due to its reaction with membrane-derived free radicals. We suspect it is primarily due to a reaction of α-tocopherol with lipid peroxidation products (see Appendix) because the depletion is significantly less when the cells are exposed to PDT in the absence of the prooxidants Fe^{2+} and ascorbate (Fig. 2).

The time course of cell death

We hypothesized that the loss of α-tocopherol and the increase in lipid radical formation from the photodynamic action of Photofrin® should result in detectable membrane damage. One measure of breached membrane integrity is the cell's inability to exclude trypan blue dye. The trypan blue assay is frequently used for measuring cell death; however, it is a direct measure of membrane damage and leakage. When L1210 cells were exposed to trypan blue after photosensitization, it was observed that they rapidly lost the ability to exclude the dye (Fig. 3). After 5 min of illumination the time at which lipid radical production slows, the percent survival as estimated by trypan blue was only about 30%.

Another technique to estimate a breach of membrane integrity and cell death utilizes flow cytometry (20). We examined the effect of photosensitization with Photofrin® on the L1210 cell using both forward scatter and PI uptake (Fig. 4). Forward scatter data depict the break-up of the cell and the resultant formation of smaller particles, *i.e.* cellular debris. The forward scatter data suggest that the cells are essentially still intact at 8 min of photosensitization, a time at which the lipid radical intensity has reached an initial plateau, trypan blue survival is below approximately 20% and α-tocopherol levels have fallen by 90%. However, at approximately 9.5 min of illumination, PI uptake begins and increases rapidly. At this same time forward scatter is in its most rapid rate of decline. We interpret this to indicate that only after nearly complete depletion of α-tocopherol, the principal small molecule membrane antioxidant, does sufficient damage occur to allow nuclear PI uptake.

Temporal overview of Photofrin® photosensitization-induced early oxidative events

To gain an overview of the events, we have normalized the data from each type of experiment and displayed the results

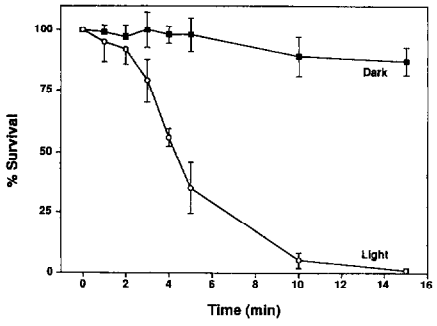

Figure 3. Trypan blue dye exclusion survival decreases with time of photosensitization. The L1210 cells, incubated with Photofrin® (15 μg/mL), were illuminated in suspension (8 × 10^6 cells/mL) in 16 × 125 mm glass screw-cap tubes in the presence of Fe^{2+} (5 μ*M*) and ascorbate (100 μ*M*) and at the same light intensity as used in the EPR experiments. At designated time points the cell suspension was subjected to trypan blue and cell survival was determined. The data represent the means and SEM of at least three independent determinations.

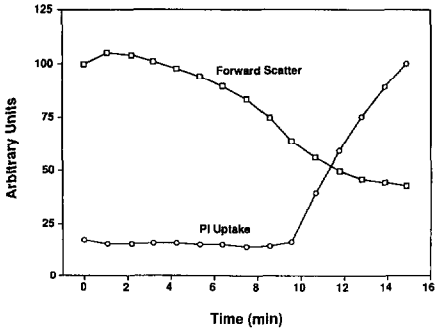

Figure 4. Photofrin®/light damage to cells as followed by flow cytometry forward scatter of light and PI uptake. The L1210 cells incubated with Photofrin® (15 μg/mL) were illuminated in suspension (8×10^6 cells/mL) in a Coulter EPICS 750 flow cytometer in the presence of Fe^{2+} (5 μ*M*) and ascorbate (100 μ*M*) and at the same light intensity as used in the EPR experiments. The PI live/dead discrimination was determined by exciting at 488 nm at 100 mW and detecting emission with a 635 nm bandpass filter. Cell debris formation was determined using forward angle light scatter.

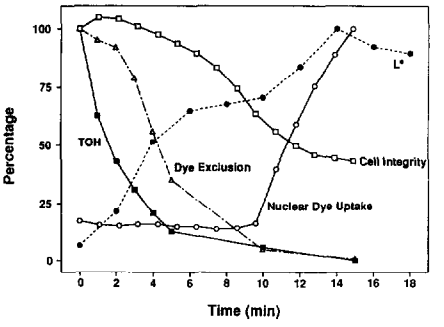

Figure 5. Superimposed sequence of oxidative events during photosensitization of L1210 cells. Shown are the kinetics of POBN/L_d· generation, cellular tocopherol (TOH) depletion, dye exclusion, nuclear PI uptake and cellular disintegration. The ordinate is percentage of baseline value (tocopherol, dye exclusion, cell disintegration) or of maximum (POBN/L_d·, PI uptake).

in Fig. 5. It is seen that lipid radicals are formed immediately upon illumination, whereas α-tocopherol levels begin an immediate and rapid rate of decline. When vitamin E is nearly depleted, cell integrity is lost. This is the sequence of events we would anticipate when cells are challenged with an oxidative stress at the membrane level. When α-tocopherol levels decline while lipid radical formation continues, we would expect substantial membrane damage to occur. Indeed the rate of membrane damage, as accessed by trypan blue, began to accelerate rapidly at the 2 min time point, the time at which α-tocopherol levels fell to below 50% of initial levels. Membrane damage can be divided into two categories based upon the severity of the damage; damage that causes a detectable breach in integrity and damage that results in physical cellular breakdown. One would expect that during an oxidative stress, such as lipid peroxidation induced by photosensitization, the cell would first become leaky before a complete loss of integrity. The forward scatter data in Fig. 5 are in agreement with this hypothesis. The forward scatter does not begin to change appreciably until the trypan blue has entered over 70% of the cells, the lipid radical concentration has reached 75% of its maximum and α-tocopherol levels have fallen by 90%. The PI assay indicates cell death by the nuclear uptake of the PI probe. The DNA interaction requires prior plasma membrane disruption. Thus the observed sequence of events is logical at both a chemical and a physical level.

In conclusion, our observations support the importance of oxidative events in Photofrin® photosensitization. Most importantly, we have observed a sequence of events which includes generation of a membrane-derived lipid radical, depletion of the major membrane antioxidant, leakage of the plasma membrane, PI staining of the nucleus and finally complete cell disruption. This suggests that the generation of the lipid radical is a seminal early event, perhaps an initial one, in PDT.

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APPENDIX

We favor the radical pathway for the loss of TOH because of a simple kinetic estimation of reaction rates. From the literature the rate constants for the reaction of $^1O_2$ with TOH and PUFA are (21)

$$k_{tq}\ (\text{TOH; EtOH}) = 3 \times 10^8\ M^{-1}\ s^{-1}$$

$$k_{cq}\ (\text{TOH; EtOH}) = 2 \times 10^7\ M^{-1}\ s^{-1}$$

$$k_{18:2}\ (\text{EtOH}) = 2.2 \times 10^5\ M^{-1}\ s^{-1}$$

(methyl linoleate)

$$k_{18:3}\ (\text{EtOH}) = 2.9 \times 10^5\ M^{-1}\ s^{-1}$$

(methyl linolenate)

$$k_{20:4}\ (C_5H_5N) = 2.9 \times 10^5\ M^{-1}\ s^{-1}$$

(methyl arachidonate)

where k_{tq} is the total quenching rate constant, physical plus chemical and k_{cq} is the chemical quenching rate constant, *i.e.* a chemical reaction that oxidizes TOH, and k_{qp} is the physical quenching rate constant. From our previous work on lipid and vitamin E content of L1210 cells, we know that the number of *bis*-allylic positions in PUFA best represents the degree of unsaturation and therefore the oxidizability of PUFA in cells (19,17,22). For our cell culture conditions we know that the *bis*-allylic position : tocopherol ratio is 2100, *i.e.* 2100 *bis*-allylic positions/1 α-tocopherol (17). We can apply this ratio directly to estimate the relative rates of $^1O_2$ reacting with the PUFA of a cell membrane compared to tocopherol. Thus

$$\frac{\text{rate } (^1O_2 + \text{PUFA})}{\text{rate } (^1O_2 + \text{TOH})} = \frac{k\ [\text{PUFA}]\ [^1O_2]}{k_{cq}\ [\text{TOH}]\ [^1O_2]} \quad \text{or}$$

$$\frac{\text{rate } (^1O_2 + \text{PUFA})}{\text{rate } (^1O_2 + \text{TOH})} = \frac{2.9 \times 10^5\ M^{-1}\ s^{-1}}{2 \times 10^7\ M^{-1}\ s^{-1}} \times \frac{2100}{1}$$

$$\frac{\text{rate } (^1O_2 + \text{PUFA})}{\text{rate } (^1O_2 + \text{TOH})} = \frac{30}{1}$$

Some of the assumptions made to arrive at this estimate are

1) the dominant chemical reactions of $^1O_2$ produced in our experimental setting are with PUFA and TOH;
2) that chemical quenching of $^1O_2$ by PUFA is the dominant reaction contributing to the rate constant $2.9 \times 10^5\ M^{-1}\ s^{-1}$ and
3) that the ratio of rate constants for chemical quenching of $^1O_2$ in EtOH holds for these same reactions in a biological membrane.

This estimate suggests that less than ≈5% of the singlet oxygen that undergoes chemical reactions will destroy TOH while more than 95% reacts with PUFA to form PUFA-OOH (LOOH). The fact that we see lipid radical products immediately, *i.e.* a buildup of POBN/$L_d^{\cdot}$, and that iron/ascorbate accelerates this free radical formation is consistent with our hypothesis that TOH is oxidized (lost) primarily by a free radical mechanism. All experiments in Fig. 5 are with iron and ascorbate present, thus free radical processes are enhanced.

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