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# Comparing β-Carotene, Vitamin E and Nitric Oxide as Membrane Antioxidants

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**Singlet oxygen initiates lipid peroxidation *via* a non-free radical mechanism by reacting directly with unsaturated lipids to form lipid hydroperoxides (LOOHs). These LOOHs can initiate free radical chain reactions leading to membrane leakage and cell death. Here we compare the ability and mechanism by which three small-molecule membrane antioxidants (β-carotene, α-tocopherol and nitric oxide) inhibit lipid peroxidation in membranes. We demonstrate that β-carotene provides protection against singlet oxygen-mediated lipid peroxidation, but does not slow free radical-mediated lipid peroxidation. α-Tocopherol does not protect cells from singlet oxygen, but does inhibit free radical formation in cell membranes. Nitric oxide provides no direct protection against singlet oxygen exposure, but is an exceptional chain-breaking antioxidant as evident from its ability to blunt oxygen consumption during free radical-mediated lipid peroxidation. These three small-molecule antioxidants appear to have complementary mechanisms for the protection of cell membranes from detrimental oxidations.**

*Key words:* Antioxidants / β-Carotene / Free radicals / Lipid peroxidation / Nitric oxide / Singlet oxygen / Tocopherol / Vitamin E.

## Introduction

Lipid peroxidation continues to be a major topic of research. Oxidation of the lipid in low density lipoproteins and associated free radical formation appears to be a major component of vascular disease (Henning and Chow, 1988; Steinberg *et al.*, 1989). The realization that free radicals are produced both in normal and pathological conditions has accelerated the search for details of the mechanisms by which various small-molecules function as effective membrane antioxidants in cells and tissues. From basic research in food and oil chemistry we have learned much about the mechanisms of lipid peroxidation (Gardner, 1989). Here we explore lipid peroxidation processes in cells. We address the differing mechanisms by which β-carotene, α-tocopherol (vitamin E) and nitric oxide serve as cellular antioxidants.

Free radical-mediated lipid peroxidation has three major components: initiation, propagation and termination (Gardner, 1989; Porter *et al*., 1996; Buettner, 1993):

$$\text{L-H} + \text{oxidant}^{\bullet} \xrightarrow{R_i} \text{L}^{\bullet} + \text{oxidant-H} \qquad \text{(initiation) (1)}$$

$$\text{L}^{\bullet} + \text{O}_2 \xrightarrow{k_o \approx 3 \times 10^8\ \text{M}^{-1}\text{s}^{-1}} \text{LOO}^{\bullet} \qquad \text{(propagation cycle) (2)}$$

$$\text{LOO}^{\bullet} + \text{L-H} \xrightarrow{k_p \approx 50\ \text{M}^{-1}\text{s}^{-1}} \text{L}^{\bullet} + \text{LOOH} \qquad \text{(propagation cycle) (3)}$$

$$\text{L}^{\bullet} + \text{L}^{\bullet} \longrightarrow \text{non-radical products} \qquad \text{(termination) (4)}$$

$$\text{L}^{\bullet} + \text{LOO}^{\bullet} \longrightarrow \text{non-radical products} \qquad \text{(termination) (5)}$$

L-H represents a lipid, generally a polyunsaturated fatty acid moiety (PUFA). (See Figure 1 legend for an explanation of the abbreviations used to denote the various chemical species associated with lipid peroxidation). The rate of the propagation event is governed by the various carbon-hydrogen bond dissociation energies along the lipid chain (Cosgrove *et al.*, 1987). The weakest carbon-hydrogen bonds are those of the *bis*-allylic methylene positions, which have carbon-hydrogen bond energies of approximately 75 kcal/mol compared to 101 kcal/mol for typical alkyl C-H bonds (Figure 2).

In homogeneous solution the oxidizability of unsaturated lipids increases linearly with the number of *bis*-allylic positions in the lipid chain (Cosgrove *et al.*, 1987). However, the rate of cellular lipid peroxidation has been shown to increase exponentially with the number of *bis*-allylic positions contained in cellular lipids (Wagner *et al.*, 1994).

### β-Carotene

β-Carotene is considered an excellent quencher of singlet oxygen (Foote and Denney, 1968; Wilkinson and Brummer, 1981). Because singlet oxygen ($^1\Delta_g O_2$ or $^1O_2$) reacts with lipids *via* a non-radical mechanism to form lipid hydroperoxides, the removal of singlet oxygen from membranes would prevent lipid peroxidation.

$$\text{L-H} + {}^1\text{O}_2 \longrightarrow \text{LOOH} \qquad (6)$$

$${}^1\text{O}_2 + \beta\text{-car} \xrightarrow{k \sim 10^{10}\ \text{M}^{-1}\text{s}^{-1}} \text{O}_2 + \beta\text{-car}^* \longrightarrow \text{O}_2 + \beta\text{-car} + \text{heat} \qquad (7)$$

Note that reaction (7) represents physical quenching of $^1O_2$ and β-carotene is not consumed. However, a small

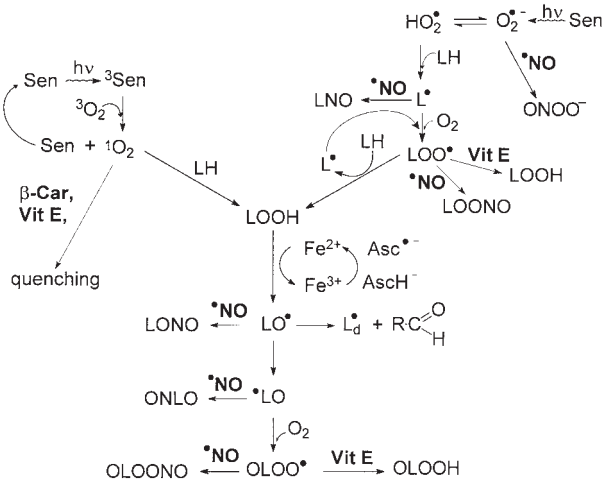


**Fig. 1** Mechanism of Photosensitizer-Induced Lipid Peroxidation.
Polyunsaturated fatty acids (LH) react with singlet oxygen to produce lipid hydroperoxides (LOOH). Ferrous iron reduces these LOOHs to lipid alkoxyl radicals (LO•). A major reaction pathway for LO• is the rearrangement to an epoxyl radical (•LO), which then reacts with oxygen to form epoxyperoxyl radicals (OLOO•). These are thought to be the major propagating species for free radical-mediated lipid peroxidation (Wilcox and Marnett, 1993; Qian *et al.,* 2000).

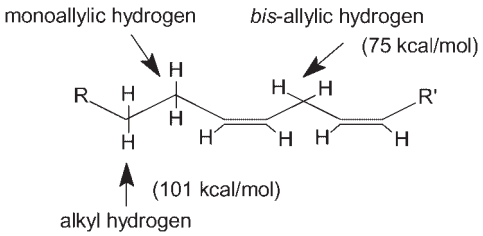


**Fig. 2** The Types of C-H Bonds in Lipids.

portion of the reactions will result in the oxidation of β-carotene. This has been estimated to be one molecule of β-carotene oxidized per 1000 molecules of $^1O_2$ quenched and perhaps as small as one in 100 000 (Handelman 1996). The asterisk indicates the excited triplet state of β-carotene.

## Vitamin E

Vitamin E has been traditionally thought to be the major non-enzymatic, small-molecule antioxidant present in the lipid structures of cells (Burton *et al.*, 1983), but it is now being realized that α-tocopherol has other specific non-antioxidant functions (Azzi *et al.,* 2001). Its characteristics as an antioxidant in solutions of fatty acids in organic solvents are well studied. In lipid solutions and lipid dispersions, it inhibits free radical-mediated lipid peroxidation until it is depleted. Upon depletion of vitamin E, lipid peroxidation accelerates, taking place at the same rate as if vitamin E had not been present (Niki *et al.*, 1984; Niki, 1987). Its inhibition of oxidation of liposomal membranes (Barclay, 1993) and lipoproteins have the same general features (Esterbauer *et al.*, 1989; Dieber-Rothenender, 1991; Bowery *et al.*, 1992). Much less is known about the action of vitamin E in cells and tissues because most previous studies of oxidation related to free radicals have been performed using model systems (micelles or liposomes), isolated LDL, or isolated cell membranes (Chow, 1991).

Vitamin E is a donor antioxidant (reductant) that will react with peroxyl radicals [see reactions (8) and (9), and also Figure 1].

(Buettner, 1993)

$$LOO^\bullet + TOH \xrightarrow{k \sim 8 \times 10^4\ M^{-1}s^{-1}} LOOH + TO^\bullet$$

(chain termination) (8)

$$OLOO^\bullet + TOH \xrightarrow{k \sim 8 \times 10^4\ M^{-1}s^{-1}} OLOOH + TO^\bullet$$

(chain termination) (9)

Thus, it serves as a chain-breaking antioxidant, inhibiting the propagation cycle of lipid peroxidation, reactions (2) and (3) above. The tocopheroxyl radical is a weak oxidant ($E^{o\prime}$=+480 mV; Buettner, 1993) that must be removed or it will initiate new free radical chain reactions (Walkdock and Stocker, 1996; Thomas *et al.*, 1997; Witting, 1997). TO• can be reduced back to TOH by ascorbate (Buettner, 1993; Sharma and Buettner, 1993):

(Bisby, 1995; Buettner, 1993)

$$TO^\bullet + AscH^- \xrightarrow{k \sim 2 \times 10^5\ M^{-1}s^{-1}} TOH + Asc^{\bullet -} \quad (10)$$

Reductive enzyme systems can also reduce TO• to TOH (Chan *et al.*, 1991). Thus, tocopherol is renewed as an antioxidant.

## Nitrogen Monoxide, alias Nitric Oxide (•NO)

Nitric oxide is a lipid-soluble molecule that can serve as a chain-terminating antioxidant during lipid peroxidation:

(Padjama and Huie, 1993)

$$LOO^\bullet \text{ or } {}^\bullet OLOO + {}^\bullet NO \xrightarrow{k \sim 2 \times 10^9\ M^{-1}s^{-1}} LOONO \text{ or } OLOONO \quad (11)$$

In these chain-termination reactions the chain-carrying peroxyl radical (LOO• or OLOO•) is removed. Typical donor antioxidants, such as vitamin E, also remove these chain-carrying radicals, reactions (8) and (9). However, it is clear that different pathways will be involved in the removal of the antioxidant radical formed in reactions (8) and (9) compared to the LOONO (or OLOONO) formed in reaction (11) (O'Donnell *et al.*, 1997). It is thought that the tocopheroxyl radical formed in reaction (8) or (9) is recycled by reductants such as ascorbate. The exact fate of LOONO and/or OLOONO is under investigation. A simple hydrolysis reaction has been proposed that will yield an alcohol and nitrate (Kelley *et al.*, 1999).

Here we have investigated the effectiveness of these three antioxidants in leukemia cells and keratinocytes exposed to singlet oxygen and/or free radical processes.

## Results

Photosensitizers can produce $^1O_2$, which results in lipid peroxidation in cells because of its reaction with unsaturated lipids forming LOOH (Thomas *et al.*, 1987; Thomas and Girotti, 1989; Lin *et al.*, 1992;). We hypothesized that membrane-associated antioxidants could prevent this singlet oxygen-induced lipid damage. However, the exact mechanism would vary depending on the chemical characteristics of the antioxidant (Figure 1).

β-Carotene, a lipid-soluble vitamin A precursor, has long been known to be an efficient singlet oxygen quencher (Foote and Denney, 1969). Evidence from bacteria, plant, animal, and human photosensitivity diseases suggest that β-carotene provides protection from the phototoxicity of both endogenous and exogenous photosensitizers by quenching singlet oxygen (Sistrom *et al.*, 1957; Mathews, 1964; Mathews-Roth, 1970). We hypothesized that β-carotene, as a singlet oxygen quencher, will decrease the steady-state concentration of singlet oxygen and thereby decrease the rate of LOOH formation and the rate of associated oxidative chemistry. To test this hypothesis we examined if increasing the β-carotene levels in membranes of human leukemia cells (HL-60) and 308 murine keratinocytes would decrease lipid peroxidation induced by the photosensitizer Photofrin and ferrous iron. We examined oxygen consumption as a measure of lipid peroxidation. Because oxygen consumption can only be conveniently measured in suspension cells, we chose HL-60 cells for these experiments. We supplemented the growth media with β-carotene and found that the cells take it up in a time- and concentration-dependent manner (Figure 3). HL-60 cells were supplemented with β-carotene for 24 h and then exposed to

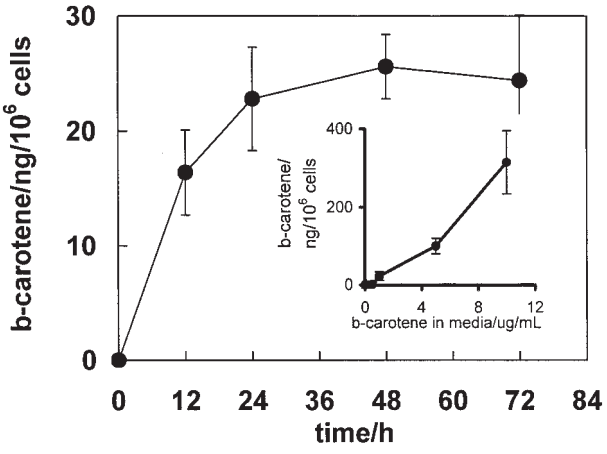


**Fig. 3** Keratinocytes Take up β-Carotene in a Time- and Concentration-Dependent Manner.
When the growth medium for the keratinocytes was supplemented with 1 μM β-carotene the amount in the cells reaches a maximum in approximately 24 h. Thus, this time was selected for experiments such as done for Figure 5. The inset shows that as the concentration of β-carotene is increased in the growth media the cellular uptake increases in an approximately linear manner.

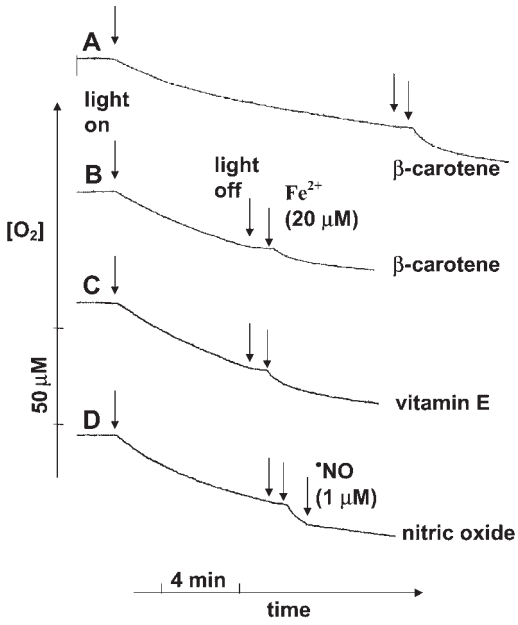


**Fig. 4** Example Oxygen Monitor Traces of HL-60 Cells Supplemented with Antioxidants.
HL-60 cells ($1\times10^7$ cells/3 ml) were grown in vitamin E (10 μM) or β-carotene (2 μM) supplemented media. Cells were then exposed to Photofrin (9 μg/ml), washed and resuspended in PBS pH 6.5 and placed into an oxygen monitor. Cells were illuminated until ~34 μM oxygen was consumed. Light was then switched off and ferrous iron (20 μM) was added to start lipid-derived free radical chain reactions. Nitric oxide (1 μM) was added 1 min after iron addition.
(A) HL-60 cells supplemented with β-carotene (1 μM). Cells were illuminated until ~34 μM $O_2$ was consumed and ferrous iron was added.
(B) HL-60 cells supplemented with β-carotene (1 μM). Cells were illuminated for 7 min; only 27 μM $O_2$ was consumed.
(C) HL-60 cells supplemented with vitamin E (10 μM). Cells were illuminated until ~34 μM $O_2$ was consumed.
(D) HL-60 cells were illuminated until ~34 μM $O_2$ was consumed. Nitric oxide was added 1 min after addition of ferrous iron. HL-60 cells were not supplemented with antioxidant in their growth media, therefore this trace can also serve as a control (before •NO addition).

Photofrin, light and then ferrous iron. When cells are exposed to singlet oxygen, oxygen is consumed. As seen in Table 1 and Figure 4, the rate of oxygen consumption during light exposure with β-carotene-treated cells is somewhat less than seen in control cells. (The experiments with HL-60 cells and β-carotene were inconsistent in that the actual rates of oxygen consumption in a set of experiments often would vary from one set to another. Comparisons were made from experiments presenting the same rate of oxygen consumption in control experiments. This aspect of these experiments needs further investigation.) Once the light is switched off the photosensitizer is no longer active and no singlet oxygen is produced; thus, the rate of oxygen consumption is near zero (Figure 4).

If catalytic metals are minimized, then, as previously

**Table 1** Oxygen Consumption and Antioxidants.

| Antioxidant | Slope, light on[a] | Plus iron slope[b] | Reduction of $O_2$ consumption by •NO after $Fe^{2+}$ |
|---|---|---|---|
| None (control) | −5.8 ± 0.3 | −11.1 ± 0.4 | N.A.[c] |
| Vitamin E | −6.3 ± 0.3 | −11.4 ± 0.4 | N.A. |
| β-Car[d] | −5.0 ± 0.5[e,f] | −10.9 ± 1.5[e] | N.A. |
| β-Car[g] | −5.2 ± 0.15[e,f] | −6.6 ± 0.3[e] | N.A. |
| •NO | −5.8 ± 0.3[h] | −11.1 ± 0.4[i] | 84%[j] |

[a] Initial slope with light on represents the rate of oxygen consumption immediately after the light is switched on (μM/min).
[b] Initial iron slope represents the rate of oxygen consumption immediately after iron addition (μM/min). Oxygen consumption before iron addition was the same for all experiments.
[c] N.A. – not applicable.
[d] In this experiment cells were illuminated until 34 μM $O_2$ was consumed.
[e]Data represent two measurements; the statistical information provides the range.
[f] In general we observed a small slowing of the initial rate of oxygen consumption with β-carotene; but when looking at longer times we saw that it takes considerably longer to consume a fixed amount of oxygen, *e.g.* 34 μM, when β-carotene is present. This suggests that the initial rate of oxygen consumption is governed by very fast reactions of $^1O_2$ that β-carotene is not able to slow effectively. However, once this phase is over, the reactions of $^1O_2$ are kinetically much slower and β-carotene is much more able to compete against these reactions. Thus, the time to consume a fixed amount of oxygen may be a better indicator of the overall effectiveness of β-carotene.
[g] In this experiment cells were illuminated for 7 min, the same time as the control cells. Only 27 μM of $O_2$ was consumed compared to 34 μM of $O_2$ in the control.
[h] There was no change in slope when •NO was added during the 'light on' phase of the experiment.
[i] This is the rate before introduction of •NO. [j] This value was estimated using the equation: [1-(•NO slope/iron slope)]×100.
Data derived from experimental conditions used as described for Figure 4. All experiments were done in PBS, pH 6.5. Data represent at least three independent experiments±SD, unless otherwise stated (see footnote[e]).

seen, one would expect that LOOHs would accumulate in the cells (Thomas and Girotti, 1989; Lin *et al.*, 1992; Buettner *et al.* 1993; Schafer and Buettner, 2000; Wang *et al.*, 2001). Addition of ferrous iron to these cells will result in the one-electron reductive cleavage of LOOH and a burst of free radical formation with a corresponding increase in the rate of oxygen consumption. Two different sets of experiments were performed. (A) Cells were illuminated until 34 μM $O_2$ was consumed. This experiment demonstrates that the rate of oxygen consumption in β-carotene cells is much slower when compared to control. To consume 34 μM $O_2$, cells with β-carotene had to be illuminated for nearly twice as long (14.5 min) compared to control cells (8 min) (see Table 1, footnote f). When the total oxygen consumption in different experiments was the same for the photosensitization phase, the rate of $O_2$ consumption upon addition of iron was the same in cells with or without β-carotene. This indicates that the β-carotene is not a significant antioxidant for the free radical processes initiated by ferrous iron. (B) In another experiment cells with β-carotene were illuminated for the same time total (7 min) as control cells. Cells with β-carotene consumed an average of 3.9 μM of oxygen per min while control cells consumed an average of 4.9 μM per min. In the β-carotene-treated cells, the rate of $O_2$ consumption after addition of iron was decreased by 40% compared to control (Table 1). These results show that β-carotene decreases singlet oxygen-induced lipid peroxidation as measured by $O_2$ consumption, but it is not an effective antioxidant for the free radical process initiated by ferrous iron.

To verify that lipid-derived radicals are formed upon addition of iron we used EPR spin trapping. Keratinocytes enriched with β-carotene were exposed to Photofrin and light. The spin trap POBN and ferrous iron were added and then the solution covering the cells that contained the spin-trapped radicals was immediately examined by EPR. A minor decrease in radical formation (statistically not significant) was observed from keratinocytes supplemented with 10 μM β-carotene (Figure 5A). A decrease in radical formation would be consistent with β-carotene scavenging the singlet oxygen produced by the photosensitizer and light, thereby lowering the level of LOOHs formed. To determine the impact of β-carotene on membrane permeability and cytotoxicity, trypan blue dye exclusion and clonogenic assays were performed. Because LOOHs are more hydrophilic than their undamaged, unsaturated lipid counterparts these molecules can disrupt membrane structure and function, thereby increasing membrane permeability. Using trypan blue dye exclusion as a measure of membrane permeability, we found that β-carotene provides no significant protection after exposure to singlet oxygen (Figure 5B). The results of clonogenic assays were comparable with the trypan blue results, suggesting only minor protection by β-carotene (Figure 5C). In summary, β-carotene, a singlet oxygen quencher, showed no significant protection from singlet oxygen induced lipid peroxidation and cyto-

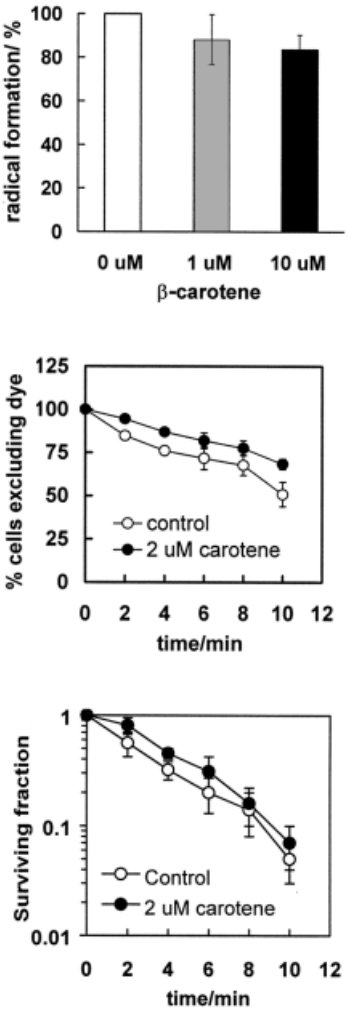


**Fig. 5** β-Carotene Provides No Significant Protection from Lipid Peroxidation Induced by Singlet Oxygen.
(A) Murine keratinocytes (308 cells) were grown in medium supplemented with 1 or 10 μM β-carotene for 24 h. For the controls, cells were incubated with 0.1% ethanol, the vehicle for β-carotene supplementation. Cells were then treated with 6 μg/ml Photofrin for 45 min. After 7 min of light exposure (5 J/m$^2$s), POBN (25 mM), ascorbic acid (100 μM) and $Fe^{2+}$ (5 μM) were added and radical formation determined by EPR. Results represent the mean of triplicate experiments. *$p$<0.05.
(B) Murine 308 cells were grown to 80% confluence in 35-mm$^2$ petridishes. Cells were incubated for 24 h with 2 μM β-carotene (in 0.1% EtOH) (●) or 0.1% ethanol (○) as control. Then cells were treated with 6 μg/ml Photofrin for 45 min in PBS pH 7.4. After light exposure, cells were trypsinized and membrane permeability was assayed by trypan blue dye exclusion. Results represent the mean of triplicate experiments.
(C) Murine 308 cells were grown to 80% confluence in 35-mm$^2$ petridishes. Cells were incubated for 24 h with 2 μM β-carotene (in 0.1% EtOH) (●) or 0.1% ethanol (○) as control. Then cells were treated with 6 μg/ml Photofrin for 45 min in PBS. After light exposure, cells were trypsinized and seeded for clonogenic assay. Results represent the mean of triplicate experiments.

toxicity in keratinocytes (adhesive cells) under our experimental conditions.

Vitamin E has been traditionally thought to be the principal small-molecule antioxidant in cell membranes, providing protection against free radical chain reactions (Burton *et al.*, 1993). Vitamin E has been shown to be a singlet oxygen scavenger, but far less effective than β-carotene $k_{quenching}$ (vitamin E in EtOH)=$10^8$ $M^{-1}s^{-1}$, while $k_{quenching}$ (β-carotene in EtOH)=$10^{10}$ $M^{-1}s^{-1}$ (Wilkinson and Brummer, 1981). However, vitamin E serves as a chain-terminating antioxidant reacting with the LOO• and OLOO• radicals ($k$=8×$10^4$ $M^{-1}s^{-1}$) (Buettner, 1993) formed during lipid peroxidation induced by singlet oxygen and ferrous iron, reactions (8) and (9). To test the influence of vitamin E on lipid peroxidation, oxygen consumption was measured in HL-60 cells exposed to Photofrin, light and ferrous iron. HL-60 cells were incubated with α-tocopherol acetate for 48 h (Kelley *et al.*, 1993) and then exposed to Photofrin for 45 min. As expected, vitamin E had no influence on the rate of oxygen consumption during singlet oxygen-induced LOOH formation (Table 1 and Figure 4).

If vitamin E terminates the lipid-derived free radical chain reaction, then the rate of $O_2$ consumption after addition of ferrous iron should be less compared to cells not supplemented with α-tocopherol acetate. However, vitamin E did not decrease the rate of $O_2$ consumption after addition of ferrous iron; addition of ferrous iron will result in lipid-derived radical formation. To confirm this result we examined lipid-derived radical formation in α-tocopherol-enriched HL-60 cells (Figure 6). HL-60 cells were supplemented with α-tocopherol acetate and then exposed to Photofrin for 45 min. Cells were washed, resuspended in 0.9% NaCl pH~6, POBN, ascorbic acid (100 μM) and then ferrous iron (5 μM) was added. Cells were immediately placed into an EPR flat cell and radical formation recorded. The first five scans were done in the dark to determine the baseline value then cells were exposed to light (180 J/m$^2$s). Under these experimental conditions LOOHs are continuously produced and subsequently reduced to form radicals. We found a dose-dependent decrease in radical formation when cells were supplemented with vitamin E (Figure 6A). When HL-60 cells or K-562 cells were exposed first to Photofrin and light to accumulate LOOH in the membranes, and then all LOOHs were reduced at essentially the same time with ferrous iron, we also found a concentration-dependent and cell line-dependent decrease in radical formation (Figure 6B; data for K562 cells are shown). However, when these experiments were done in PBS (final pH 6.5 or 7.5) or NaCl (final pH 6.5) vitamin E had little impact on radical formation (data not shown). Thus, experimental conditions are important when determining the effect of antioxidants. Our observations are consistent with there being a threshold level of α-tocopherol (~10 μM) that will provide protection against free radical formation in cells. This threshold level is parallel to that observed by Wagner *et al.* (1996) in L1210 cells and consistent with the notion that tissue culture cells are deficient in vitamin E (Kelley *et al.*, 1995). Supplementation of culture media with tocopherol acetate increases the level of vitamin E in cells, but the level in

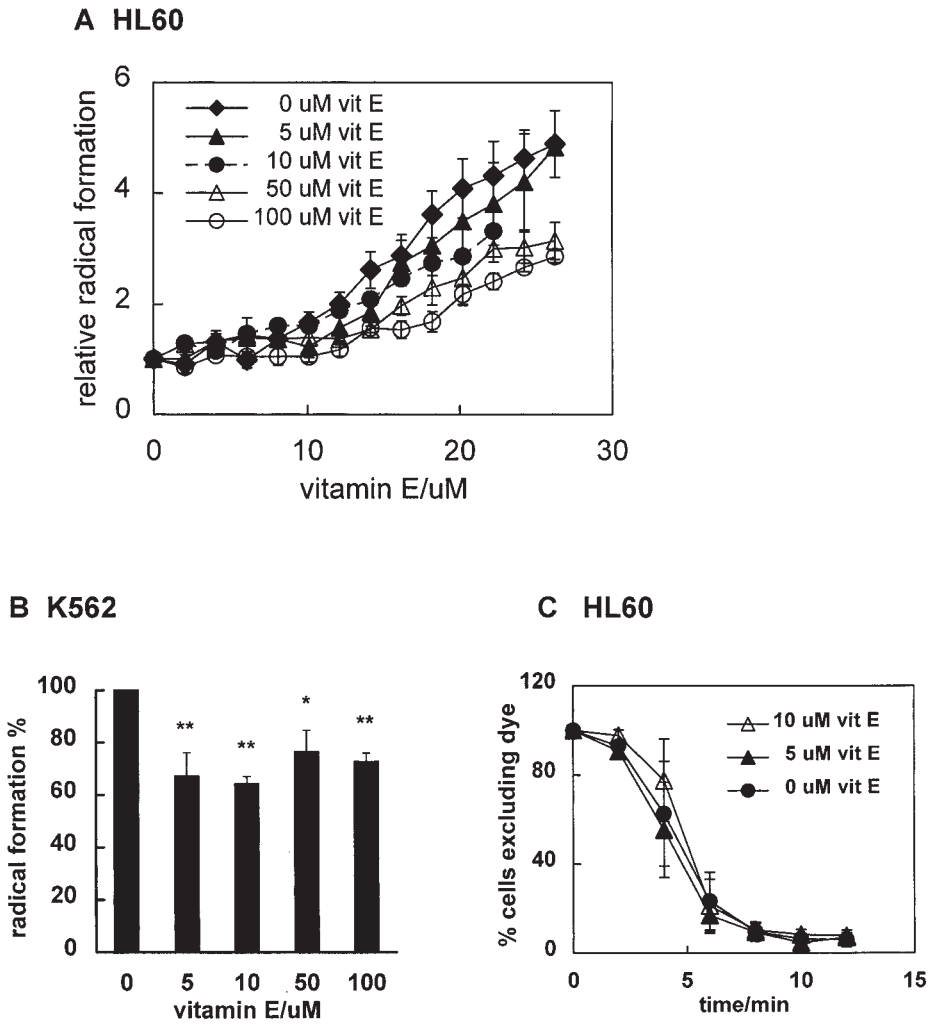


**Fig. 6** Vitamin E Reduces Lipid-Derived Radical Formation in Cells Exposed to Photofrin and Light.
EPR signal intensity of POBN/$L_d^{\bullet}$ produced from HL-60 cells and K-562 cells ($8\times10^6$ cells/ml), treated with α-tocopherol acetate (5 – 100 μM) for 48 h and then Photofrin (9 μg/ml) for 45 min.
(A) An aliquot of cells (500 μl) in 0.9% NaCl, pH~6, was mixed with POBN (25 mM), ascorbic acid (100 μM) and ferrous iron (5 μM) and placed into a TM EPR quartz flat cell. Cells were exposed to visible light (tungsten, 180 J/m$^2$s) and the POBN radical adduct EPR signal intensity was monitored *versus* time. Each data point is a ratio of the EPR signal height at time *t* min with the signal height at time zero. Each spectrum is the signal-averaged result of 5 scans of the low field doublet of the POBN/lipid-derived radical adduct spectrum. The first five scans, representing time zero, were performed in the dark.
(B) K562 cells were resuspended in 0.9% NaCl, POBN (25 mM) was added and cells illuminated (180 J/m$^2$s) for 2 min. During light exposure, LOOHs accumulate in the cell membranes. Upon addition of ascorbate (100 μM) and $Fe^{2+}$ (5 μM) a burst of radical formation was detected by EPR spin trapping. EPR instrument settings were: 1.0 G modulation amplitude, $1\times10^6$ receiver gain; 40 mW nominal power; 5 scans were averaged.
(C) Trypan blue dye exclusion from HL-60 cells ($4\times10^6$ cells/ml) treated with α-tocopherol acetate (48 h) and Photofrin (9 μg/ml). Cells were resuspended in PBS, pH 7.4; ascorbate (100 μM) and ferrous iron (5 μM) were added and cells exposed to light (5 J/m$^2$s). Dye exclusion was determined every 2 min with a light microscope.
Statistically significant values with *$p<0.05$ or **$p<0.01$ compared to respective control. During tocopherol acetate supplementation control cells were exposed to an appropriate aliquot of 100% EtOH solution as vehicle control.

cells saturates with increasing media concentration of vitamin E.

We further examined the ability of α-tocopherol to protect against membrane damage induced by Photofrin and light. Independent of Photofrin dose (9 or 0.9 μg/ml) or cell line (HL-60 or K-562), vitamin E did not protect against singlet oxygen-induced membrane permeability. Figure 6C shows an example experiment. Our observations on both membrane damage and radical formation with vitamin E are comparable to the results with β-carotene.

Nitric oxide may play a role in the photodynamic therapy of cancer (Korbelik *et al.*, 1997 and 1998). It has been reported that tumors that produce high levels of •NO are in general more resistant to photodynamic therapy (Thomsen *et al.*, 1995). We have observed that PDT, using

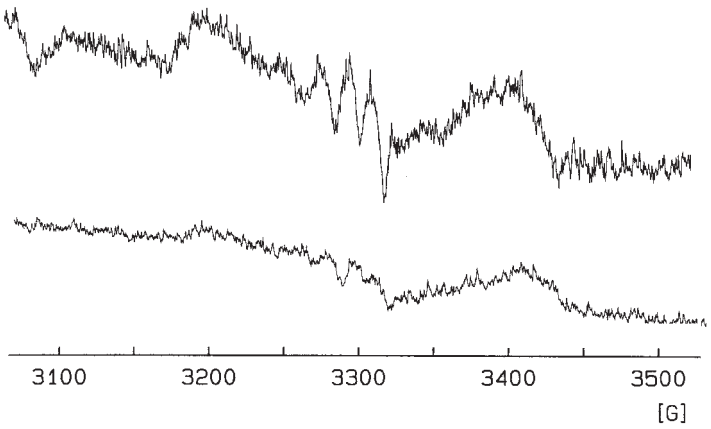


**Fig. 7** Nitrosyl-Heme EPR Signals Are Seen in Tumor Tissue Subjected to PDT.
DBA/2 mice, bearing vascularized L1210 solid tumors in the hind limb, were subjected to Photofrin (*i.p.*) and illuminated with visible light. After illumination tissue biopsies were collected and immediately examined by EPR, 77 K. A significant increase in •NO-heme adduct is observed.
Upper panel: EPR spectrum from tumor treated with Photofrin and light. This EPR spectrum shows the characteristic 3-line signal from a nitrogen hyperfine splitting ($a^N$=17.5 G; g=2.0012) of a 5-coordinate Fe(II)-heme with •NO as the fifth ligand axial to the heme plane.
Lower panel: EPR spectrum from tumor treated with light only (without Photofrin). All controls gave only background levels of •NO-heme as seen in the lower spectrum. Controls included: no Photofrin; no light; muscle tissue, liver tissue and not illuminated tumor tissue located in the opposite hind limb of the same animal undergoing full treatment.
Spectra are the result of signal-averaging. The different S/N levels are a result of a differing number of scans. The spectra shown were normalized to the same number of scans allowing direct comparison of signal heights.

Photofrin, induces •NO production in murine tumors. DBA/2 mice, bearing tumors, were subjected to Photofrin® and visible light. EPR spectra from biopsies of these tumors after illumination revealed the presence of •NO-heme adducts (Figure 7). These data lead us to conclude that PDT induces production of •NO in a tumor-specific and not a systemic manner. The nitric oxide produced during PDT could be a pro-oxidant but could also serve as chain-terminating antioxidant during lipid peroxidation. Nitric oxide, being quite lipid soluble, has been proposed to be a more effective small-molecule antioxidant in cell membranes than vitamin E because of its favorable kinetics for the reaction with radicals formed during lipid peroxidation. Vitamin E will predominantly serve as a chain-breaking antioxidant reacting with LOO• or OLOO• ($k$=8×10$^4$ M$^{-1}$s$^{-1}$) (Buettner, 1993). However, it is estimated that •NO will react ~10 000 times faster with these radicals ($k$=2×10$^9$ M$^{-1}$s$^{-1}$) (Padjama and Huie, 1993; O'Donnell *et al.*, 1997; Kelley *et al.*, 1999).

We have shown previously that •NO can inhibit iron-induced lipid peroxidation in cells (Kelley *et al.*, 1999). This observation prompted us to ask if •NO could inhibit singlet oxygen-induced lipid peroxidation. We measured oxygen uptake, using a Clarke electrode, to monitor lipid peroxidation in HL-60 cells. Illumination of cells treated with Photofrin and light resulted in a rapid uptake of oxygen by the cells. Introduction of •NO (1.0 μM) during light exposure resulted in no change in the rate of oxygen consumption, data not shown. This result suggests that these low levels of •NO have no effect on singlet oxygen or on the photosensitizer. Addition of ferrous iron after light exposure initiated another phase of rapid uptake of oxygen due to radical reactions. Introduction of •NO (1.0 μM) after ferrous iron addition inhibited oxygen consumption up to 84% (Table 1 and Figure 4). These results demonstrate that low physiological levels of •NO have no influence on the non-free radical chemistry that results from singlet oxygen exposure, but •NO is an especially effective small-molecule antioxidant that can protect cells from free radical-mediated oxidation processes.

## Discussion

Lipid peroxidation is implicated in various diseases and is often initiated during disease treatments; such as photodynamic therapy of cancer. There is still much to learn about the mechanism of antioxidant protection in lipid peroxidation. We investigated the influence of three small-molecule antioxidants with different chemical characteristics on lipid peroxidation. All three antioxidants have in common that they are lipid soluble and therefore accumulate in cell membranes. β-Carotene is a well-established singlet oxygen quencher that could slow $^1O_2$-induced oxygen uptake from Photofrin and light. Vitamin E is a membrane antioxidant with some $^1O_2$ scavenging abilities, but its main antioxidant characteristic is thought to be as a donor antioxidant, reacting with peroxyl radicals forming hydroperoxides. Nitric oxide is a relatively long-lived radical that can react in principle with all lipid-derived radicals. We found that these three antioxidants afford different protection against cellular lipid peroxidation induced by Photofrin and light.

When comparing the results of the three small molecule antioxidants β-carotene, vitamin E and nitric oxide, we found that in HL-60 cells:

(i) β-carotene slowed $^1O_2$-induced oxygen-uptake from Photofrin and light, preventing the formation of LOOHs, while neither vitamin E nor nitric oxide altered the rate of oxygen consumption in these cells when exposed to singlet oxygen.
(ii) Vitamin E is able to slow the rate of radical formation during a continuous oxidative challenge (Figure 6A). However, it provided no protection from singlet oxygen exposure (Figure 4A, Table 1) or from a burst of free radical formation resulting from exposure of $^1O_2$ exposed-cells to ferrous iron (Table 1). Vitamin E provided no protection against membrane permeability (Figure 6C). This is not surprising as LOOH formation *via* $^1O_2$ may be principally responsible for the change in membrane integrity; vitamin E, as a less efficient singlet oxygen scavenger, cannot reduce the levels of LOOH formed *via* $^1O_2$.
(iii) Nitric oxide at low physiological concentrations •NO is a highly effective chain-breaking antioxidant in cells. It does not influence $^1O_2$-mediated processes, but rather slows free radical-mediated lipid peroxidation as measured by $O_2$ consumption (Table 1 and Figure 4). The results presented here clearly demonstrate the efficacy of nitric oxide as an antioxidant during iron-induced free radical chain processes. Neither β-carotene nor vitamin E came close to offering this level of protection from free radical processes. Thus, we propose that •NO is an important, as yet not appreciated, membrane antioxidant in cells.

Each of these small-molecule antioxidants fill a niche. Although not addressed directly here, they appear to complement each other and in some ways serve as a team providing protection from unwanted membrane oxidations.

## Materials and Methods

### Chemicals

Photofrin® (porfimer sodium) was kindly provided by QLT Phototherapeutics, Inc., Vancouver, BC, Canada. Phosphate buffered saline (PBS): 210 mg potassium dihydrogen phosphate, 407 mg sodium monohydrogen phosphate and 9 g sodium chloride (Fisher, Fair Lawn, USA) were dissolved in 1 l water and adjusted to pH 7.4 or 6.5 with 1 M HCl. Sodium chloride solution (0.9% sodium chloride) and the PBS solution were stored over chelating resin (Sigma, St. Louis, USA) to minimize the level of adventitious transition metals. α-(4-Pyridyl-1-oxide)-N-*tert*-butylnitrone (Aldrich, Milwaukee, USA) was prepared as a 1 M stock solution in distilled water. Ascorbic acid (10 mM) and ferrous iron (1 mM) stock solutions were prepared in distilled water just before each experiment. α-Tocopherol acetate and trans β-carotene was purchased from Sigma.

### Cell Culture

Human leukemia cells, HL-60 and K-562, and the murine leukemia cells L1210 were acquired from American Type Culture Collection (ATCC). K-562 and L1210 cells were grown in medium consisting of RPMI 1640 medium (Life Technologies, Rockville, USA), 10% fetal bovine serum (FBS), Penicillin (100 U/ml) and Streptomycin (10 μg/ml), while HL-60 cells were grown in the same medium supplemented with L-glutamine (2 mM). Cells were grown in antibiotic-free medium one passage before experiment. Murine 308 keratinocytes were acquired from Dr. Frederick Domann (University of Iowa) and grown in MEM-α medium (Life Technologies, Rockville, USA) supplemented with 10% FBS. Experiments were performed in exponential growth phase.

### Vitamin E and β-Carotene Supplementation

Stock solutions of α-tocopherol acetate (5–100 mM) in 100% EtOH were made weekly and stored at 4°C. Cell medium was supplemented with α-tocopherol acetate (0–100 μM) and cells incubated for 48 h. The α-tocopherol acetate is taken up by cells and converted to α-tocopherol. A stock solution (suspension) of β-carotene (1 mM) in 100% EtOH was prepared and stored at –20°C. Cell medium was supplemented with β-carotene (0, 1, 2 or 10 μM) and cells incubated for 24 h. We found that uptake of β-carotene by cells is time and concentration dependent. Maximum uptake of β-carotene (as determined with HPLC) is observed to occur after 24 h under our experimental conditions, similar to our observations with vitamin E (Kelley *et al.*, 1995; Wagner *et al.*, 1996). As a vehicle control, an appropriate aliquot of 100% EtOH was added to the cell suspension for both vitamin E and ß-carotene supplementation experiments.

### β-Carotene Uptake

The uptake of β-carotene in cells was measured by high-performance liquid chromatography (HPLC) with a UV/Vis detector. After β-carotene treatment, cells were extracted using hexane:2-propanol (3:2, *v/v*). The hexane layer was collected and dried under nitrogen. The residue was resuspended in 100 μl hexane and injected into a Beckman 110 A HPLC with a Waters 8MB C18 column (10 μm particle size, 5×10 mm). The sample was eluted using hexane:methanol (98:2, *v/v*) as the mobile phase at a flow rate of 2 ml/min. The effluent was monitored at 450 nm by a Gibson Holochrome UV/VIS detector. The amount of cellular β-carotene was calculated from a standard curve.

### Nitric Oxide Delivery

Nitric oxide was added as a bolus to the cell solutions. Nitric oxide gas was obtained from a •NO gas tank (99.5% •NO, AGA Speciality Gas, Maumee, USA). The •NO gas was purified and the •NO stock solution was prepared as previously described (Venkataraman *et al.*, 2000). The concentration of •NO in the stock solution was determined using a Sievers 280 Nitric Oxide Analyzer (NOA), Boulder, USA. To deliver the •NO solution, a gas-tight syringe was flushed with nitrogen gas. Then ~10 μl $N_2$ were injected into the container to replace the volume of the •NO stock solution to be withdrawn. Next, ~10 μl •NO solution were drawn into the syringe and injected into the oxygen monitor sample chamber containing the cell suspensions.

### Photofrin

Photofrin (3 mg/ml) was dissolved in 5% dextrose (pH 7.4), sterile filtered, aliquoted and frozen at –20°C. Cells were exposed to Photofrin (9, 6 or 0.9 μg/ml) at 37°C for 45 min in PBS (pH 7.4). After incubation with Photofrin, cells were washed and resuspended in PBS or 0.9% NaCl. This protocol results in Photofrin being predominantly in the plasma membrane of cells (Boehmer and Morstyn, 1985).

### Light Source

Two different light sources were used:

(a) For the oxygen monitor and EPR experiments a tungsten lamp emitting visible light focused on the center of the sample was used (typically 350 or 180 J/m$^2$s on the sample).
(b) For the trypan blue and clonogenic experiments a light box equipped with a circular Sylvania light bulb (daylight, 22 W) covered with a light diffuser that is impermeable to UV was used (≈5 J/m$^2$s on the sample).

Light intensity was measured with a Yellow Spring Instrument 65 A Radiometer with the 6551 Probe (Yellow Springs, USA). All samples were placed in the same marked positions on the light box that provided an consistent fluence rate.

### Membrane Permeability and Clonogenic Assay

Trypan blue dye exclusion was used to determined membrane permeability while clonogenic assay were used to determine cytotoxicity. Murine 308 cells were grown to 80% confluence in 35-mm$^2$ petri dishes. Cells were incubated for 24 h with 2 μM β-carotene (in 0.1% EtOH) or 0.1% ethanol as control. Then cells were treated with 6 μg/ml Photofrin for 45 min in PBS. After light exposure (5 J/m$^2$s), cells were trypsinized and membrane permeability was assayed by trypan blue dye exclusion or seeded and grown for 12 days for clonogenic assay. For the vitamin E experiments HL-60 or K-562 cells (4×10$^6$ cells/ml) were treated with α-tocopherol acetate (48 h) and 9 or 0.9 μg/ml Photofrin (45 min). Cells were washed and resuspended in PBS or NaCl. Ascorbate (100 μM) and $Fe^{2+}$ (5 μM) were added and cells exposed to light (5 J/m$^2$s). Trypan blue dye exclusion was determined.

For the clonogenic assay cells were treated with β-carotene (2 μM), Photofrin and light. Keratinocytes were then trypsinized and counted. A certain number of cells were seeded into 35-mm dishes and incubated at 37°C for 12 days to allow colony formation. Colonies were fixed with acetic acid and methanol (3:1, v/v), and stained with 0.1% crystal violet. Colonies containing more than 50 cells were counted. Cell survival was calculated as:

Surviving fraction = number of colonies formed/(number of cells seeded) × plating efficiency.

### EPR Experiments

EPR experiments were performed with a Bruker EMX EPR spectrometer (Karlsruhe, Germany). Murine keratinocytes (308 cells) were grown in medium supplemented with 2 or 10 μM β-carotene for 24 h. Cells incubated with 0.1% ethanol were used as control. Cells were then treated with 6 μg/ml Photofrin for 45 min, resuspended in PBS pH 7.4 and illuminated for 7 min (5 J/m$^2$s). POBN (25 mM), ascorbic acid (100 μM) and ferrous iron (5 μM) were added. The PBS mixture was transferred to an EPR flat cell and the POBN radical adduct EPR signal intensity monitored. Ascorbic acid was added to recycle ferric iron to ferrous iron. Radical formation was normalized to total cell number. Results represent the mean of triplicate experiments.

HL-60 cells (8×10$^6$/ml) were incubated with α-tocopherol acetate (0–100 μM) for 48 h, then incubated with Photofrin (9 μg/ml) for 45 min, washed and resuspended in 1 ml 0.9% NaCl. Two different experiments were conducted:

(a) An aliquot of 500 μl cell suspension was mixed with POBN (25 mM), ascorbic acid (100 μM) and ferrous iron (5 μM) and placed into a TM EPR quartz flat cell. Cells were exposed to visible light (tungsten, 180 J/m$^2$s) and the POBN radical adduct EPR signal intensity was monitored *versus* time. In Figure 6A, each data point is a ratio of the EPR signal height at time t min with the signal height at time zero min. Each spectrum is the signal-averaged result of 5 scans of the low field doublet of the POBN/lipid-derived radical adduct spectrum. The first five scans, representing time zero, were performed in the dark.
(b) An aliquot of a 500 μl cell suspension (K-562 cells) was placed in a reagent tube, POBN (25 mM) added and cells were exposed to visible light (tungsten bulb, 180 J/m$^2$ s) for 2 min. Then, ascorbic acid (100 μM) and ferrous iron (5 μM) were added and the sample placed into a TM EPR quartz flat cell. Five scans of the low field doublet of the POBN/lipid-derived radical adduct spectrum were collected.

### Oxygen Consumption

Oxygen consumption was measured using a YSI $O_2$-monitor with a Clarke electrode as a measure for lipid peroxidation. HL-60 cells were grown in medium with/without β-carotene (0–2 μM) or α-tocopherol acetate (0–10 μM) and then incubated with Photofrin (9 μg/ml) for 45 min. Cells (1×10$^7$ cells/3 ml) in PBS pH 6.5 were placed into an oxygen monitor. Cells were exposed to light (typically tungsten 350 J/m$^2$s) until approximately 34 μM $O_2$ were consumed. Then light was switched off to inactivate the photosensitizer. After 1 min in the dark, ferrous iron (20 μM) was added to start lipid-derived radical chain reactions. Then approximately 1 min later $^{\bullet}$NO (0 or 1.0 μM) was added. For the 0 μM $^{\bullet}$NO experiments, the same volume of a $N_2$-purged aqueous solution was added.

### PDT *in vivo*

L1210 murine leukemia cells were passaged *via* ascities in female DBA/2 mice. Upon harvest the cells were centrifuged and resuspended at 2×10$^6$ cells/ml in 0.9% NaCl. This cell suspension (0.1 ml) was injected subcutaneous into the hind limb of female CDF1 mice. After approximately 7 days a palpable tumor was observed and at day 14 most tumors had reached 0.5–0.8 cm in diameter. Pathological analysis of control L1210 solid tumors displayed substantial vasculature. At this time, the mice were injected with 25 mg/kg (*i.p.*) Photofrin. After 24 h the skin covering the tumor was removed; the tumor was bathed in 0.9% NaCl and illuminated with visible light from a tungsten source, filtered to remove infrared; the tumor temperature never exceeded 36°C. EPR spectra of biopsies from the tumors were collected at 77 K using a finger-dewar in a standard 3 mm quartz tube.

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