Chapter 49

Oxidative Stress and Antioxidant Intervention

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Keywords: Antioxidants, free radicals

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Critical Reviews of Oxidative Stress and Aging: Advances in Basic Science, Diagnostics and Intervention. (2003) Ed Richard G. Cutler and Henry Rodriguez. World Scientific, New Jersey, London, Singapore, Hong Kong. Volume II. Chapter 49, pp 849-869.
ISBN 981-02-4636-6

1. Introduction

The term antioxidant is now a part of the American vocabulary. Antioxidant formulations are widely available in health food stores, pharmacies, supermarkets and through mail-order sources. These formulations range from the traditional antioxidant vitamins C and E, to phenolic antioxidants isolated from various plant sources, e.g. pycnogenol, xanthohumol, green tea extracts and other polyphenols. Producers and vendors suggest that their products lead to better health. Only a few of the claims have been supported by sound research. Other than for antioxidant vitamins, many claims of health benefits of antioxidants are extrapolations from experiments that simply demonstrate that a substance can serve as an antioxidant.

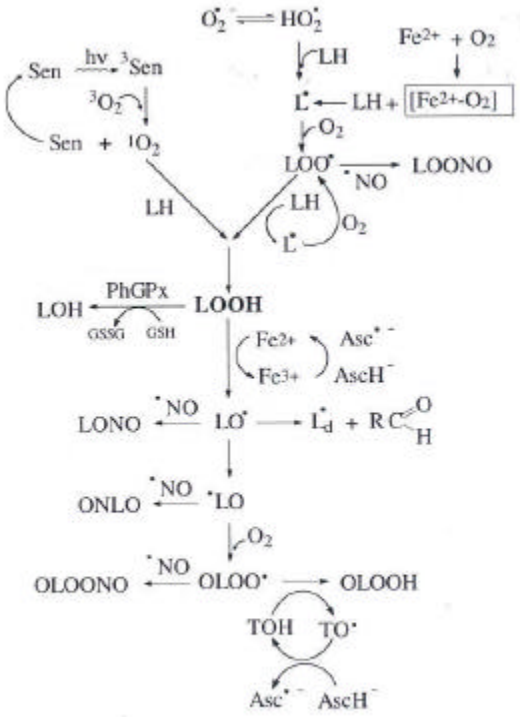
An antioxidant is a substance that, when present in small amounts, prevents oxidation of the bulk. Unfortunately, many compounds are inappropriately labeled as antioxidants. Many experiments have been reported to demonstrate a purported antioxidant reaction of a substance, but in reality the data only suggest a slight retarding reaction.[1] There is still much to learn about phytochemicals and anti-oxidant action.

A good deal is known about traditional antioxidants such as vitamin E and vitamin C.[2-4] Many investigators have examined the antioxidant action of these two small molecules using cell culture models as well as animal models. The results clearly demonstrate that these antioxidants protect cells and tissues from oxidative stress. They suggest that in stress situations, such as disease or exercise, intake of higher amounts of these antioxidants would be beneficial. But direct demonstration that higher than normal intakes of these antioxidants is beneficial is experimentally difficult. In this work we discuss the mechanisms of how antioxidants work together and look at examples that demonstrate this cooperativity.

2. Lipid Peroxidation and Antioxidants

There are many types of antioxidants; they can be classified by their mechanism of action. Preventative antioxidants include peroxide decomposers and metal ion decomposers, while chain-breaking antioxidants intercept chain-carrying radicals. Many chain-breaking antioxidants donate a hydrogen atom to the chain-carrying radical thereby stopping the oxidation process. This results in an antioxidant radical. However, this radical is much less reactive than the original chain-carrying radical. But even this much more domesticated radical must be removed. Tocopherol (TOH) is a typical donor antioxidant in this class; it protects against lipid peroxidation, Scheme 1. Lipid peroxidation is a chain reaction, reaction 1-3.

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



Scheme 1. **Lipid peroxidation and antioxidants.**

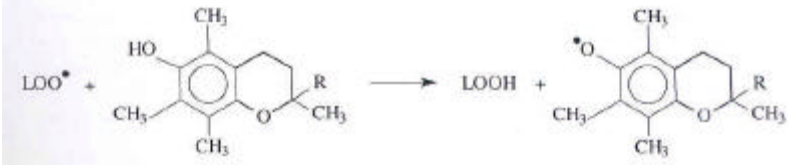
$$L^{\bullet} + O_2 \xrightarrow{k_0=3\times10^8\ M^{-1}s^{-1}} LOO^{\bullet} \quad \text{(propagation cycle)} \quad (2)$$

$$LOO\bullet + LH \xrightarrow{k_p=10\text{-}50\ M^{-1}s^{-1}} L\bullet + LOOH \quad \text{(propagation cycle)} \quad (3)$$

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

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

Tocopherol breaks the propagation cycle by donating a hydrogen atom to the chain-carrying peroxyl radical, $LOO^{\bullet}$, thereby stopping the oxidation process, reaction 6.



(6)

The tocopheroxyl radical ($TO^{\bullet}$) is much less reactive than LOO•, but it too can be a chain-carrying radical, albeit very poorly, reaction 7.

$$TO^{\bullet} + LH \xrightarrow{k_p=0.05\ M^{-1}s^{-1}\ [5,6,7]} L^{\bullet} + TOH. \qquad \text{(propagation)} \qquad (7)$$

The tocopheroxyl radical can be repaired by additional donor compounds that are thermo-dynamically more reducing than TOH.[8,9] Examples are ascorbate ($AscH^-$) or ubiquinol ($CoQH_2$), see Table 1.

The reaction of either $AscH^-$ or $CoQH_2$ with $TO^{\bullet}$ naturally results in formation of a radical, $Asc^{\bullet-}$ or $CoQ^{\bullet-}$, respectively. These radicals are in turn removed by enzyme systems; thus $AscH^-$ and $CoQH_2$ are recycled.

Table 1. One-Electron Reduction potentials

Redox Couple	$E^{\circ\prime}$/mV
$HO^{\bullet}, H^+/H_2O$	+2310
$RO^{\bullet}, H^+$/ROH (aliphatic alkoxyl radical)	+1600
$ROO^{\bullet}, H^+$/ROOH (alkyl peroxyl radical)	+1000
$GS^{\bullet}/GS^-$ (glutathione)	+920
$PUFA^{\bullet}, H^+$/PUFA-H (bis allylic-H)	+600
$TO^{\bullet}, H^+$/TOH	+480
$H_2O_2, H^+/H_2O, HO^{\bullet}$	+320
$Ascorbate^{\bullet-}, H^+$/Ascorbate monoanion	+282
Semiubiquinone, H^+/ubiquinol ($CoQ^{\bullet-}$, $2H^+/CoQH_2$)	+200
Fe(III) EDTA/Fe(II) EDTA	+120
Ubiquinone, H^+/Semiubiquinone ($CoQ/CoQ^{\bullet-}$)	-36
$O_2/O_2^{\bullet-}$	-160[a]
$Paraquat/Paraquat^{\bullet+}$	-448
Fe(III)DFO/Fe(II)DFO	-450
$RSSR/RSSR^{\bullet-}$(GSH)	-1500
H_2O/e^-_{aq}	-2870

[a] Two different thermodynamic reference states are used for O_2. Here we have chosen to use the aqueous concentration of oxygen, thus the appropriate reference state is a solution that is 1 molal (= 1 M) and $E^{\circ\prime}$ = –160mV. The second reference state often used is 1 atmosphere of O_2; $E^{\circ\prime}$ is then –330 mV. A pressure of 1 atmosphere of O_2 will result in $[O_2] \cong 1.25$ mM in room temperature squeous solutions. If this reference state is used, then in the Nernst equation P_{O2} replaces $[O_2]$ in all equations. The same value for E will result. Table derived from Ref. 31.

3. Vitamin E

3.1. Vitamin E Levels in Cells can be Manipulated

We have found that most cultured cells are deficient in vitamin E.[10] L1210 cells, cultured in standard growth media, contain only 2.3 ± 0.03 μg of tocopherol/ 10^8 cells, table 2. When these cells are transplanted and grown for the same time in the ascites fluid of mice fed standard diets, the vitamin E content of the cells increases to 5.8 ± 0.06 μg of α-tocopherol/10^8 cells, a level lower than would be expected if the cells were grown in fully vascularized compartments. This apparent tocopherol deficiency in cultured cells is likely due to the low concentrations of tocopherol contained in most tissue culture media, even with the addition of serum.

To study this apparent deficiency and the relationship of cellular tocopherol to the cellular lipid composition, we supplemented the growth media of murine leukemia cells (L1210) with α-tocopherol and compared the resultant cellular tocopherol content to the degree of unsaturation of cellular lipids (membrane lipid *bis*-allylic hydrogen positions). The α-tocopherol was incorporated by cells in a time- and concentration-dependent manner with plateaus at 24 h and 100 μM, respectively. A maximum 400% increase in cellular tocopherol was easily achieved.

By experimentally modifying the fatty acid content of cellular lipids, we were able to determine that cellular tocopherol uptake and content is not a function of cellular lipid composition. Cells enriched with polyunsaturated lipids incorporated tocopherol to the same extent as those enriched with more saturated lipids. Thus, as the cellular polyunsaturated fatty acid content increases the tocopherol:

Table 2: α-tocopherol and *bis*-Allylic Positions in L1210 Cells

Cell Lipid Modification[a]	TOH Molecules per Cell ($x10^7$)[b]	*bis*-Allylic Positions per Cell ($x10^{11}$)[c]	*bis*-Allylic per TOH[d,e]
Unmodified	3.28	0.68	2075
18:1	2.78	0.25	899
18:3	3.08	2.07	6710
20:4	2.68	2.93	10 990
22:6	3.00	2.97	9900

[a]The lipid profile of cells was modified by supplementing the culture media with the indicated fatty acid. This manipulation allows us to vary the oxidizability of the cell. No supplemental tocopherol was provided to these cells.

[b]This is the number of tocopherol molecules per L1210 cell (SE = approximately 1).

[c]This is the number of *bis*-allylic positions in the lipids in an L1210 cell. It is the *bis*-allylic positions of the lipid that are susceptible to oxidation (SE = approximately 0.1).

[d]This ratio indicates the average number of lipid *bis*-allylic positions that each TOH must protect from oxidation. The greater the number the less protection afforded by TOH.

[e]Data are derived from Ref. 10.

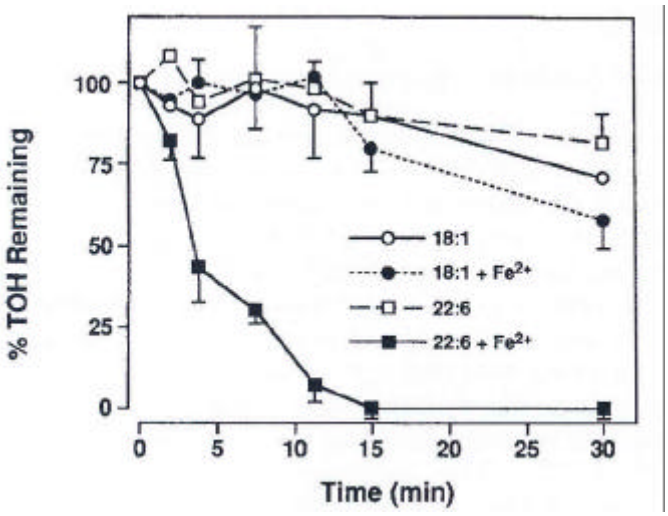


Fig. 1. **Effect of oxidative stress on cellular** a**-tocopherol.** L1210 cells (5 x 10^7) were enriched with 22:6 or 18:1 by adding 32 μM of the fatty acid to the growth medial for 48 hours. During the final 24 h, 12.5 μM tocopherol acetate was added. The enriched cells were washed and then incubated in 0.9% NaCl with or without 100 mM Fe^{2+} and the cellular tocopherol determined at the time points shown. Shown are the levels as a percentage of time zero and values are the mean and SE of three determinations. The values at time zero were: 18:1, 1.84 ± 0.6 mg/10^8 cells; 18:1 + Fe^{2+}, 1.48 ± 0.4 μg/10^8 cells; 22:6, 1.70 ± 0.1 μg/10^8 cells; and for 22:6 + Fe^{2+}, 2.0 ± 5.0 μg/10^8 cells.

bis-allylic position ratio in the cells decreases, resulting in less antioxidant protection for each lipid double bond. It is the *bis*-allylic moieties in lipids that are susceptible to oxidation. When polyunsaturated fatty acid-enriched cells are exposed to an oxidative stress, the rate of oxidation is high and the tocopherol levels decline much faster than in cells enriched with saturated fatty acids. In effect, they have much less antioxidant protection. This loss of tocopherol correlates with the *bis*-allylic:tocopherol ratio, Fig. 1. Modifying the lipid content of cells, as well as their a-tocopherol content, affects their susceptibility to free radical-mediated lipid peroxidation.

To further investigate the effect of vitamin E supplementation on lipid peroxidation in L1210 murine leukemia cells,[11] cells were exposed to an oxidative stress induced by 20 μM Fe^{2+} and 100 μM ascorbate. The kinetics of the generation of lipid-derived free radicals, as measured by EPR spin trapping (a product) and O_2 consumption (a reactant) were measured. Cells grown for 24 h with supplemental vitamin E (5 – 100 μM) in their media had a slower rate of lipid radical generation compared to cells grown without vitamin E supplementation. This inhibition in the rate of oxidation was generally dependent upon the amount of vitamin E supplementation, Fig. 2.[11] In complementary studies measuring O_2

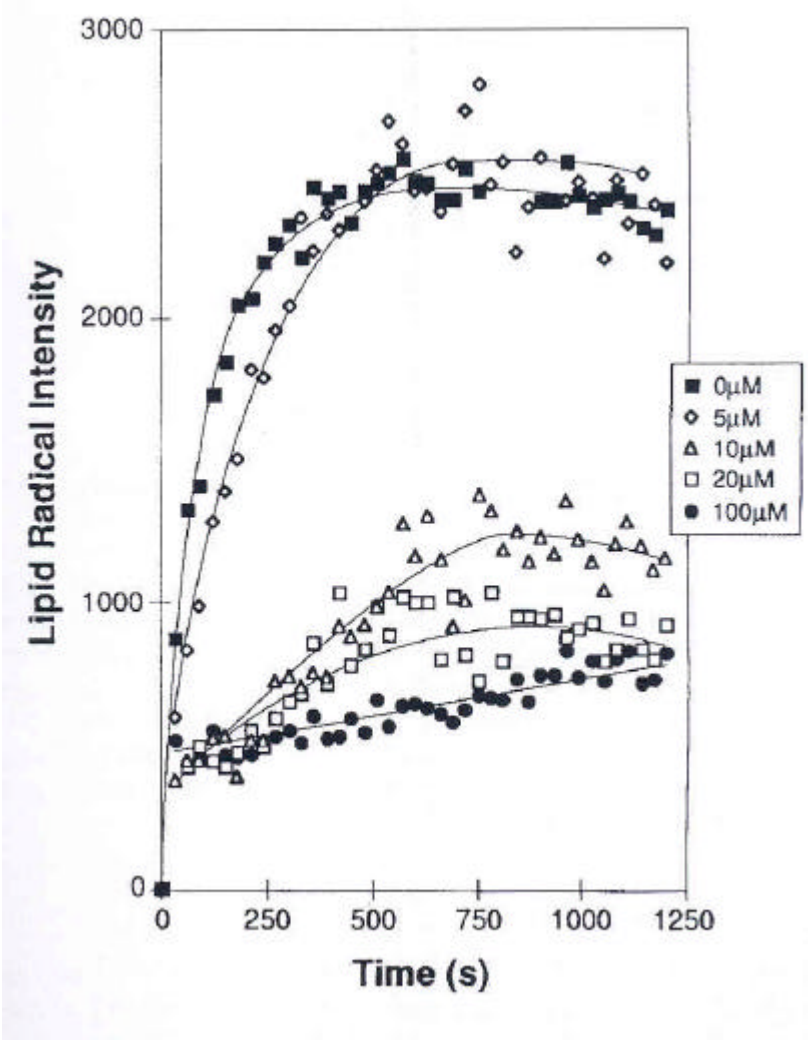


Fig. 2. **Vitamin E slows the rate of cellular lipid radical formation.** Cellular incorporation of α-tocopherol slows the rate, but induces no apparent lag phase in lipid radical formation during lipid peroxidation as shown by EPR-detectable POBN/L$^{\bullet}$ adducts. L1210 cells, 5x 10^6 cells/mL enriched with 22:6ω3 and supplemented with various concentrations of α-tocopherol acetate were subjected to the oxidative stress presented by 20 μM Fe^{2+} and 100 μM ascorbate in the presence of 50μM of the spin trap POBN. Each data point is the mean of 5 – 7 experiments and represents the EPR peak height of the first peak of the low field doublet. The lipid radical intensity units of the ordinate are arbitrary values; 1000 corresponds to 0.24 μM POBN/L$^{\bullet}$.

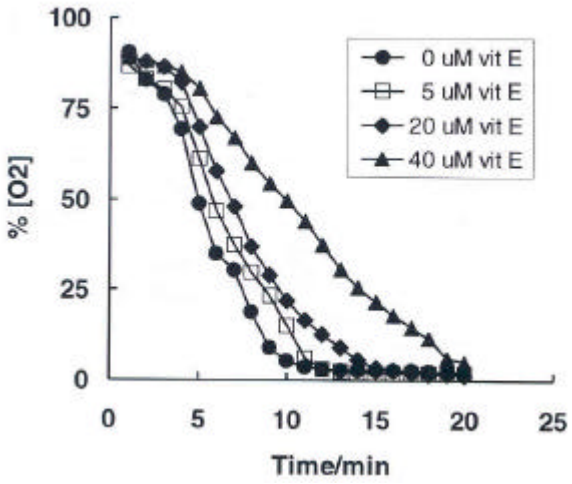


Fig. 3. **Vitamin E slows the rate of oxygen consumption during cellular lipid peroxidation.** Cellular incorporation of vitamin E slows the rate of oxygen consumption during cellular lipid peroxidation. L1210 cells (5 x 10^6 cells/mL) enriched with 22:6ω3 and supplemented with various concentrations of α-tocopherol acetate were subjected to the oxidative stress presented by 20 μM Fe^{2+} and 100 μM ascorbate in the presence of 50 mM POBN. Each point is the mean of 5 – 7 experiments from oxygen probe recordings. The conditions for these incubations were identical to those described in the legend of Fig. 2. The incubations were initially air-saturated, which implies the dissolved [O_2] = 250 μM. The POBN was included to maintain identical conditions compared to the spin trapping experiments of Fig 2. However, there was little if any difference between oxygen-uptake experiments with or without POBN.

Consumption, 5 – 100 μM vitamin E slowed the rate of oxidation (10-fold with 100μM supplemental vitamin E) consistent with the EPR studies, Fig. 3.[1]

Figure 4 clearly shows the relationship of cell tocopherol content, rate of oxygen consumption, rate of lipid radical formation, and trypan blue dye exclusion.[11] Note that cellular membranes appear to "saturate" with vitamin E. Only about 10 μM of vitamin E in the media is required; the antioxidant protection provided by this vitamin E also "saturates."

These results are the first to actually demonstrate, by monitoring free radical production, that vitamin E inhibits lipid peroxidation in cells by slowing the rate of lipid peroxidation.

3.2 In Vivo Photoprotection by Vitamin E

If an organism is undergoing an oxidative stress, then tocopherol may provide some protection from this stress. We investigated the photoprotective effect of

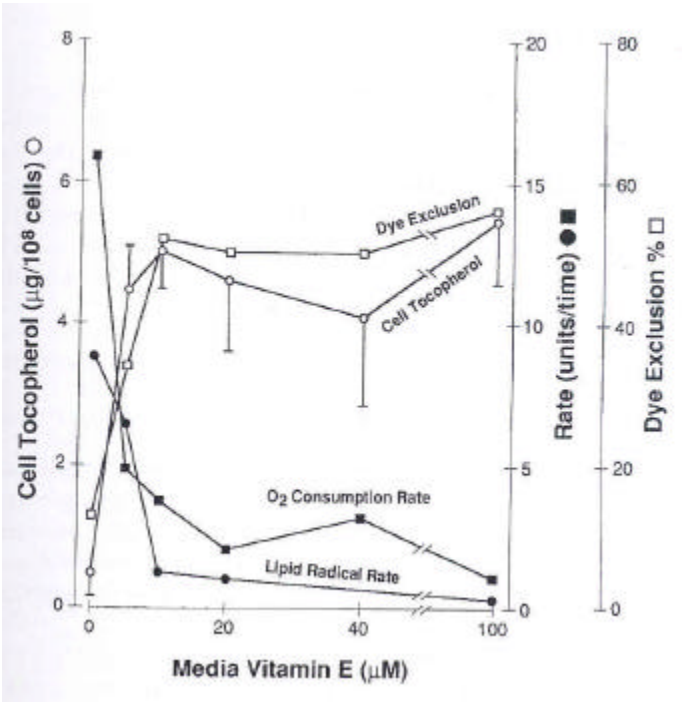


Fig. 4. **Effect of vitamin E concentration on inhibition of cellular lipid peroxidation.** The ordinate shows the initial rate of generation of POBN/L$^{\bullet}$ (derived from Fig. 2), (negative) initial rate of oxygen consumption (Fig. 3), trypan blue dye exclusion, and cellular uptake of vitamin E. The abscissa is the concentration of α-tocopherol in the culture media.

topically applied tocopherols against chronic UVB radiation-induced skin damage, using a mouse model of photoaging.[12,13] In previous testing using this model,[14] tocopherol acetate was poorly photoprotective against chronic UVB radiation-induced skin wrinkling, whereas a-tocopherol provided significant protection. We compared the efficacy of these two materials against tocopherol sorbate.[15] The results demonstrated that tocopherol sorbate is significantly more protective than the other two forms of vitamin E against skin wrinkling. In addition we found that the mice in the tocopherol sorbate group (1.8 tumors/mouse) and the α-tocopherol group (2.0 tumors/mouse) had fewer tumors compared to the vehicle control group (3.6 tumors/mouse) at the end of the study (week 23). Tocopherol sorbate and α-tocopherol reduced the average number of tumors per mouse, but they did not delay the onset of appearance of the first tumor, relative to vehicle. Tocopherol if delivered appropriately, can provide significant protection from UV-induced skin damage and tumor formation.

4. Nitric Oxide as an Antioxidant

Vitamin E (TOH) is an important lipid-soluble antioxidant and has been traditionally thought to be the principal chain-breaking antioxidant in blood and in lipid structures of cells.[16,17] However, the experiments done that led to this conclusion were done in the absence of $^{\bullet}NO$.

Nitric oxide can protect cells against the detrimental effects of reactive oxygen species.[18.19] Using low density lipoprotein as well as model systems, it has been demonstrated that $^{\bullet}NO$ can serve as a chain-breaking antioxidant to blunt lipid peroxidation.[20-23]

$$LOO^{\bullet} + {}^{\bullet}NO \longrightarrow LOON{=}O \quad \text{(chain-termination)} \quad (8)$$

$$LO^{\bullet} + {}^{\bullet}NO \longrightarrow LON{=}O. \quad \text{(chain termination)} \quad (9)$$

To test the hypothesis that $^{\bullet}NO$ can serve as a chain-breaking antioxidant in cell membranes, we examined the effect of $^{\bullet}NO$ on iron-induced lipid peroxidation in human leukemia cells.[24] We exposed HL-60 cells (enriched with docosahexaenoic acid, DHA) to an oxidative stress (20μM Fe^{2+}) and monitored the consumption of oxygen as a measure of lipid peroxidation. While oxygen consumption in our cellular vitamin E experiments slowed with increasing vitamin E levels (Fig. 3), oxygen consumption was completely arrested by the addition of $^{\bullet}NO$, Figs. 5 and 6. The duration of inhibition of oxygen consumption by $^{\bullet}NO$ was concentration-dependent in the 0.4-1.8μM range. The inhibition ends upon depletion of $^{\bullet}NO$. but note that once lipid peroxidation is under control only nM levels of $^{\bullet}NO$ are required to stop subsequent free radical chain reactions; these levels are in the physiological range.

4.1 A Theoretical Comparison of $^{\bullet}NO$ and Vitamin E as Cell Membrane-Antioxidants[24]

From the data above and kinetic information from the literature, it is possible to make an estimate of the importance of TOH versus $^{\bullet}NO$ as a chain-breaking antioxidant. A typical TOH level in cells is 1000-20000 PUFA/1 TOH.[26] If PUFA constitutes about 22% of the lipids in a cell membrane,[27] then there are about 5000-10,000 total fatty acid chains/1 TOH. As a first approximation, the mole fraction of TOH in lipid regions of membranes will be:

$$\begin{aligned} \text{Mole fraction (TOH)} &\approx 1/ (5000 \text{ to } 10\,000) \\ &\approx 1 / 7500 \\ &\approx 1.3 \text{ x } 10\text{-}4. \end{aligned}$$

Because TOH and the various fatty acids have similar properties, we assume for simplicity, that they have the same partial molar volume in the lipid regions of

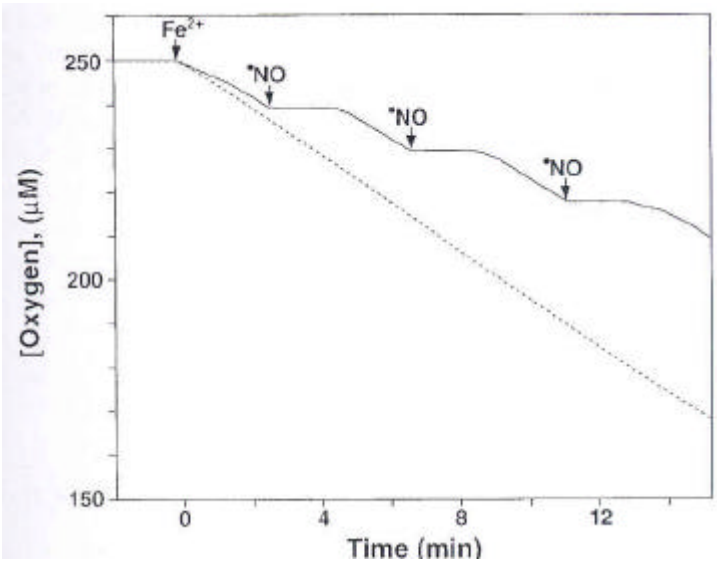
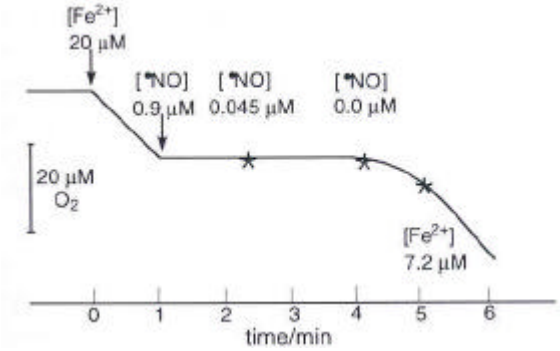


Fig. 5. **Nitric oxide inhibits iron-induced lipid peroxidation.** The rate of O_2 consumption of HL-60 cells (5 x 10^6/mL) enriched with docosahexaenoic acid was determined using a YSI oxygen monitor. Fe^{2+} (20 μM) was added at the first arrow and subsequently •NO (1.8 μM) was added (other arrows). When •NO was added, the O_2 consumption was inhibited for a period of a few minutes, then it resumed at near its initial rate until the reintroduction of additional •NO. Also shown (lower dashed line) is a typical control of



HL-60 cells subjected to Fe^{2+}-induced oxidative stress in the absence of •NO addition.

Fig 6. **Changes in concentration of • and Fe^{2+} during cellular lipid peroxidation and its inhibition by •NO.** Shown are the concentrations of • and Fe^{2+} at key time points. Peroxidation was initiated with 20 μM Fe^{2+}. At 1 min after the addition of Fe^{2+}, 0.9 μM •NO was introduced. Nitric oxide was rapidly depleted and is below the limit of detection at about 4 minutes. At the time of •NO depletion, rapid O_2 uptake resumes. This re-initiation of O_2 consumption is due to Fe^{2+} that is still present at 7.2 μM or about 36% of its original value.

cell membranes. Using a density of ≈ 0.9 g lipid/mL and an average molecular weight of 300 g/mole for the fatty acyl chains of the lipids in a cell membrane, the effective molarity of the fatty acyl chains in the lipid regions of membranes will be ≈ 3 M. The molarity of TOH will then be about 3 M x 1.3 x 10^{-4} ≈ 400 mM.

The rate at which •NO would terminate the chain propagation reactions in lipid peroxidation by reacting with the chain-carrying peroxyl radical, LOO•, reaction 8, will be:

$$\text{rate inhibition by } {}^{\bullet}\text{NO} = k_{NO}\,[{}^{\bullet}\text{NO}][\text{LOO}^{\bullet}] \tag{10}$$

where k_{NO} = 2 x 10^9 $M^{-1}s^{-1}$ [28] and [•NO] = 8 x 45 nM = 360 nM. Here 8 is the estimated membrane/water partition coefficient for •NO,[29,30] 45 nM is the measured •NO concentration in the aqueous phase of the cell suspension during the inhibition phase of lipid peroxidation, Fig. 6. So,

$$\text{rate inhibition } ({}^{\bullet}\text{NO}) = 2 \times 10^9\ M^{-1}\ s^{-1} \times 360 \times 10^{-9}\ M\ [\text{LOO}^{\bullet}] \tag{11}$$

The rate at which TOH would terminate these reactions, reaction 6, would be:

$$\text{rate inhibition (TOH)} = k_{TOH}\,[\text{TOH}][\text{LOO}^{\bullet}] \tag{12}$$

where k_{TOH} = 8 x 10^4 M^{-1} s^{-1} [31] and [TOH] = 400 mM. Thus, rate inhibition (TOH) = 8 x 10^4 M^{-1} s^{-1} x 400 x 10^{-6} M [LOO•]. The ratio of these rates is:

$$\text{rate } ({}^{\bullet}\text{NO})/\text{rate (TOH)} = 20/1 \tag{13}$$

If the concentrations and kinetic parameters used to make this estimate accurately represent these processes in cells, then an aqueous concentration of •NO of only 2 nM would provide antioxidant protection for membrane lipids equal to that typical of vitamin E. This is a remarkably low concentration of •NO and is easily achievable in many cells and tissues. That these hypotheses have merit is the recently published observation that •NO spares vitamin E in liposomes.[32] In addition these investigators demonstrated that •NO does not react with tocopherol or its radical. Nitric oxide has been shown to protect against the cytotoxicity of oxidative stress in endothelial cells, fibroblasts, hepatocytes, intestinal epithelium, and cardiomyocytes.[33-38] However, there is much yet to be learned about the molecular mechanisms of this protection and the important of •NO as an antioxidant in cells and tissues. •NO is a remarkable antioxidant.

5. Vitamin C

5.1. Ascorbate, the Terminal Small-Molecule Antioxidant

Ascorbate, an excellent reducing agent, undergoes two consecutive, reversible, one-electron oxidation processes forming the ascorbate radical ($Asc^{\bullet-}$) as an

intermediate. $Asc^{\bullet-}$ has its unpaired electron in a highly delocalized π-system making it a relatively unreactive free radical. These properties make ascorbate a superior biological, donor antioxidant.[39] $Asc^{\bullet-}$ can be detected in biological fluids or tissues using electron paramagnetic resonance spectroscopy (EPR). This is consistent with ascorbate's role as the terminal small-molecule antioxidant, Table 1.[40] As seen in Table 1, ascorbate has a low reduction potential. Thus, it can repair many free radicals that are produced during oxidative stress, such as $HO^{\bullet}$, $RO^{\bullet}$, $LOO^{\bullet}$, $GS^{\bullet}$, urate, and even the tocopheroxyl radical ($TO^{\bullet}$). The electron transfer reaction:

$$AscH^{-} + X^{\bullet} \rightarrow Asc^{\bullet-} + ZH \qquad (14)$$

is relatively rapid.[41] Consequently, both thermodynamically and kinetically, ascorbate can be considered to be an excellent aqueous antioxidant.

The ascorbate radical formed in these reactions is relatively stable and does not react with O_2 to form dangerous peroxyl radicals. Ascorbate (most likely Asc^{2-}, and/or $Asc^{\bullet-}$) appears to produce very low levels of superoxide. [42,43] Superoxide dismutase provides protection from this possibility.[44] In addition, $Asc^{\bullet-}$ as well as the dehydroascorbic formed can be reduced back to ascorbate by enzyme systems. Hence, this antioxidant is recycled.

5.2. Ascorbate can be a Pro-Oxidant

Ascorbate is known to be and is widely used as a pro-oxidant.[45-53] This behavior results because it is an excellent reducing agent. As a reducing agent it is able to reduce catalytic metals such as Fe^{3+} and Cu^{2+} to Fe^{2+} and Cu^{+}. The redox cycling of these metals is essential to the oxidation of the vast majority of singlet state organic molecules.[54] In nearly all the experimental systems where ascorbate has pro-oxidant properties, there is the simultaneous presence of redox active metals. Thus, in living organisms catalytic metals and ascorbate are to be avoided.

On the other hand, ascorbate in combination with catalytic metals are used to induce a controlled, well defined oxidative stress, such as that used in the experiments of Figs. 3 and 4. The reduced metal can react with peroxides (Fenton reaction) forming hydroxyl or alkoxyl radicals, which are very oxidizing and will initiate biological damage.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{-} + HO^{\bullet} \qquad \text{Fenton Reaction} \qquad (15)$$

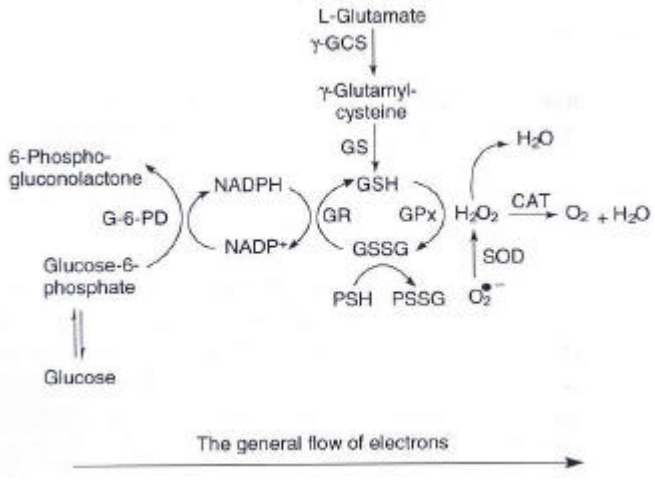
$$Fe^{2+} + ROOH \rightarrow Fe^{3+} + OH^{-} + RO^{\bullet} \qquad \text{Organic Fenton Reaction} \qquad (16)$$

However, peroxides are not necessary for these reduced metals to be dangerous. For example, in the biological pH range, all that is required for Fe^{2+} to initiate detrimental oxidations is the presence of dioxygen.[55] It is thought that $Fe^{2+} + O_2$

results in the formation of iron-oxygen complexes that are oxidizing species; the reactivity of the iron in this chemistry depends upon its coordination environment. The reactivity of iron is also influenced by pH. Using a tissue culture model, it has been observed that the lower the extracellular pH, the higher the flux of free radicals induced by the addition of ferrous iron and the greater the cellular membrane damage, as determined by trypan blue exclusion.[56] The presence of reducing agents such as ascorbate will, in general, recycle the catalytic iron, ensuring that it will be dominantly in the ferrous state. Thus, ascorbate in the presence of iron can be detrimental rather than beneficial.

6. Antioxidant Network

Oxidative stress has many faces. There are a variety of reactive oxygen species producing unwanted oxidations in different locations. Each antioxidant has its niche in that each provides protection in distinct environments and against certain types of species. To provide optimal protection from unwanted oxidations, a network of antioxidants is needed. Many of these antioxidants work together, i.e. they are co-antioxidants. They recycle each other or work in concert.



Scheme 2. **A subset of the antioxidant network.** This schematic shows the relationships between antioxidant enzymes and glutathione. Abbreviations: CAT = catalase; G-6-PD = glucose-6-phosphate dehydrogenase; γ-GCS = γ-glutamylcysteine synthetase; GS = glutathione synthetase; GPx = glutathione peroxidase; GR = glutathione reductase; PSH = protein thiol; PSSG = protein mixed disulfide with GSH; SOD = superoxide dismutase.

For example, glutathione recycles not only vitamin C[57,58] but also glutathione peroxidase (GPx) an antioxidant enzyme known to reduce H_2O_2. GP_x itself works in concert with another antioxidant enzyme, superoxide dismutase (SOD). SOD reacts with superoxide radical producing hydrogen peroxide, Scheme 2. These few examples show clearly the network that exits between various antioxidants *in vivo*.

Gey re-examined epidemiological data to see if the nutritional antioxidants vitamins E and C, are indeed antioxidants.[59] Vitamin E can protect LDL, cell membranes, and other lipid structures from oxidation. However, the resulting tocopheroxyl radical must be removed, preferably recycled. Ascorbate can do this. If this cooperativity is important, then there might be an ideal level of these co-antioxidants for optimal health. Gey found that, assuming sufficiency of both E and C, the data suggested that a ratio of C to E in plasma (i.e. [vitamin C]/[vitamin E]) of 0.6–0.8 associates with increased risk of coronary heart disease, whereas a ratio > 1.3–1.5 may be desirable to minimize risk of this disease. This is consistent with these antioxidant vitamins serving as co-antioxidants, providing conditions for optimal health.

7. Aging

The free radical theory of aging would suggest that keeping a full complement of antioxidants may be of benefit for better health and increase the probability of reaching one's maximum lifespan potential. Nutritional antioxidants have an important role in health. They work together with the endogenous antioxidants such as glutathione. Measurements of glutathione in plasma show a general decline with age.[60] Specifically, the half-cell reduction potential of the GSSG/2GSH couple in plasma becomes more positive with age, which suggests that as we age free radical oxidation processes increase.[61,62].

8. Summary

Antioxidants are necessary for life. We have a network of small-molecule and enzyme antioxidants that work together to defend against unwanted, detrimental oxidations. New, quantitative viewpoints suggest that optimal health is associated with a "healthy" redox state. A shift in the redox state to more positive potentials is detrimental to cells, tissues and whole organisms.[63] The role of antioxidants may be to protect the structure and function of the machinery that maintains a healthy redox status. Thus, we propose that studies on antioxidants and health should include quantitative measures of redox state as an indicator of overall organism health.

9. Abbreviations

$AscH^-$, ascorbate monoanion; $Asc^{\bullet-}$, ascorbate radical; Asc^{2-}, ascorbate dianion; $CoQH_2$, ubiquinol; $CoQ^{\bullet-}$, semiubiquinone; DFO, deferrioximine; DHA, docosahexaenioc acid; EPR, electron paramagnetic resonance; Fe^{2+}, ferrous iron; Fe^{3+} ferric iron; GSH, glutathione; GSSG, glutathione disulfide; GPx, glutathione peroxidase; HL-60, human leukemia cells; H_2O_2, hydrogen peroxide; $^{\bullet}OH$, hydroxyl radical; $L^{\bullet}$, lipid radical; LDL, low density lipoprotein; LH, unsaturated lipid; $LOO^{\bullet}$, lipid peroxyl radical; LOOH, lipid hydroperoxide; L1210, murine leukemia cells; $^{\bullet}NO$, nitric oxide; O_2, dioxygen; $O_2^{\bullet-}$, superoxide radical; PhGPx, phospholipid hydroperoxide glutathione peroxidase; POBN, α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone; PUFA, polyunsaturated fatty acid; $RO^{\bullet}$, aliphatic alkoxyl radical; SOD, superoxide dismutase; TOH, tocopherol; $TO^{\bullet}$, tocopheroxyl radical; XH, substrate; $X^{\bullet}$, substrate radical.

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