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Brief Communication

INTERACTION OF VITAMIN C AND VITAMIN E DURING FREE RADICAL STRESS IN PLASMA: AN ESR STUDY

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Abstract—To study the interaction of the antioxidant vitamins C and E in a biological system, we used electron spin resonance (ESR) spectroscopy to make serial measurements of ascorbate and tocopheroxyl free radicals in plasma subjected to continuous free radical-mediated oxidative stress. Upon initiation of a continuous oxidative stress, we observed an immediate increase in the concentration of ascorbate radical, which reached a peak, and then steadily declined. Only after the virtual disappearance of the ascorbate radical did we observe the appearance of the tocopheroxyl radical. These data are consistent with the hypothesis that ascorbate is the terminal small-molecule antioxidant in biological systems. This is the first experimental demonstration that the predicted thermodynamic hierarchy of ascorbate, α-tocopherol, and their free radicals holds in a biological system containing endogenous levels of these antioxidant vitamins.

Keywords—Ascorbate, Tocopherol, Free radical, Electron spin resonance

INTRODUCTION

Vitamins C and E are widely recognized as naturally occurring antioxidants in biological systems. There is intriguing evidence that these vitamins, acting as antioxidants, have a role in the prevention of cardiovascular atherosclerosis. Human epidemiological studies have shown an inverse correlation between the blood levels of vitamins C and E and the risk of ischemic heart disease.[1,2] Vitamin E has been shown to prevent/retard spontaneous, as well as experimental, atherosclerosis in fat-fed animals.[3–6] Free radical-mediated oxidation of low-density lipoproteins is now believed to be a key factor in the production of atherosclerosis, which can be effectively prevented by vitamin E.[7–9]

In addition, it has been hypothesized that vitamins C and E work synergistically to protect lipids from peroxidation.[10] When plasma is subjected to free radical-mediated oxidative stress, there is a steady decline in the level of ascorbate.[11,12] Only after the complete consumption of ascorbate does the α-tocopherol concentration begin to fall, coinciding with the appearance and steady increase in the plasma lipid hydroperoxide content.[12] Pulse radiolysis experiments have demonstrated that in homogeneous solution vitamin E will react with peroxyl free radicals;[13] the vitamin E radical (tocopheroxyl radical) thus formed can subsequently be repaired through the one-electron oxidation of vitamin C, thereby regenerating vitamin E.

$$R^{\bullet} + O_2 \longrightarrow ROO^{\bullet}$$

$$ROO^{\bullet} \rightleftharpoons TOH \rightleftharpoons A^{\bullet -}$$
$$ROOH \quad TO^{\bullet} \quad AH^{-}.$$

To our knowledge, neither the actual production of ascorbate ($A^{\bullet -}$) and tocopheroxyl ($TO^{\bullet}$) free radicals nor their temporal characteristics have been demonstrated in a biological fluid subjected to oxidative stress. We have used electron spin resonance (ESR) spectroscopy to study the production and temporal relationship of ascorbate and tocopheroxyl radicals in plasma subjected to continuous free radical-mediated oxidative stress.

EXPERIMENTAL PROCEDURES

Plasma was separated from heparinized human blood. A continuous flow pump was used to circulate

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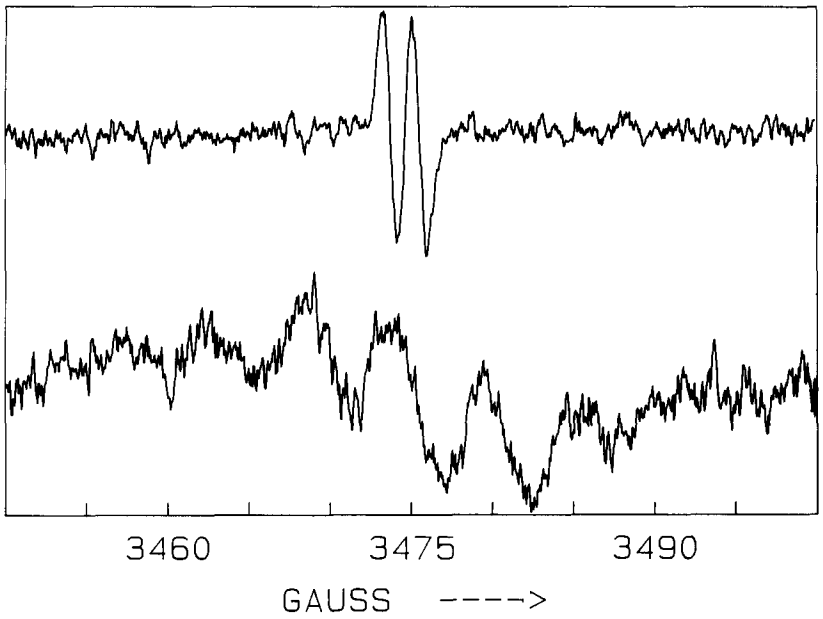

Fig. 1. ESR spectra of ascorbate and tocopherol free radicals observed in plasma subjected to oxidative stress. *TOP:* Morphology of the ascorbate radical spectrum (a^H = 1.8 G) collected at time point 1.5 min in Fig. 2. Instrument settings: modulation amplitude 0.96 gauss; scan rate 50.0 gauss/168 s; time constant 0.66 s; receiver gain 5×10^6. *BOTTOM:* Morphology of tocopheroxyl free radical spectrum collected at time point 70 min in Fig. 2. Instrument settings: modulation amplitude 0.96 gauss; scan rate 50 gauss/335 s; time constant 1.3 s; receiver gain 5×10^6.

plasma from a single donor through an ESR aqueous flat cell so as to provide constant aeration. The circulating plasma sample was continuously scanned by ESR for the presence of ascorbate and tocopheroxyl radicals. Free radical-mediated oxidative stress was produced by adding hypoxanthine (0.5 mM) and xanthine oxidase (10 mU/ml) to the sample. At these concentrations, the rate of superoxide production was estimated to be $\approx$ 70 nM/s using cytochrome *c* and ultraviolet (UV)-visible spectroscopy.[14,15]

A Bruker (Karlsruhe, Germany) ESP-300 ESR spectrometer with a TM_{110} cavity was used to collect all electron paramagnetic resonance (EPR) spectra. Quantitation of the two radicals was carried out using 3-carboxyproxyl as a standard, as outlined by Buettner,[16] after accounting for saturation effects.[17]

RESULTS AND DISCUSSION

Plasma was continuously scanned for ascorbate and tocopheroxyl free radicals by ESR spectroscopy (see Fig. 1). Prior to the introduction of the free radical oxidative stress, ascorbate free radical (a^H = 1.8 G) was observed at very low concentrations. This low steady-state level of $A^{\bullet -}$ is consistent with slow on-going oxidations in the plasma.[16,18-20] However, upon the introduction of a superoxide radical-generating system (hypoxanthine plus xanthine oxidase), there was an immediate increase in the intensity of $A^{\bullet -}$ (Fig. 2).

This increase in the steady-state $A^{\bullet -}$ concentration indicates that the rate of free radical reactions has indeed increased in the plasma and that ascorbate is serving as an antioxidant.[19] No such increase was observed with hypoxanthine alone or xanthine oxidase alone. With the continuing free radical-initiated oxidative stress, the concentration of $A^{\bullet -}$ decreases over time. In plasma there is no enzyme system to recycle oxidized ascorbate, i.e., to reduce dehydroascorbic or ascorbate radical back to ascorbate.[11,12] Thus, as reduced ascorbate is depleted, the steady-state concentration of $A^{\bullet -}$ will decrease, as observed in Figure 2.[19]

Only after the virtual disappearance of the ascorbate radical did the tocopheroxyl free radical appear in the ESR scans. As with $A^{\bullet -}$, the steady-state $TO^{\bullet}$ concentration progressively increased to a maximum, and then gradually declined, as expected with the depletion of reduced tocopherol (Fig. 2). In other words, as long as ascorbate is available to reduce (recycle) $TO^{\bullet}$ back to fully functional tocopherol, the steady-state $TO^{\bullet}$ concentration remains sufficiently low to escape detection by ESR. However, when all of the ascorbate is consumed, the steady-state concentration of $TO^{\bullet}$ will increase to an ESR detectable level. After the depletion of reduced ascorbate, there will be a gradual depletion of tocopherol. Thus, after pro-

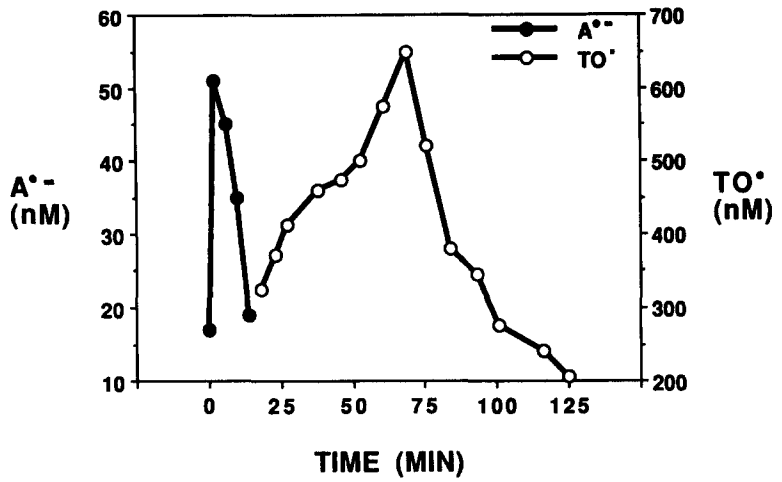

Fig. 2. Ascorbate (●) and tocopheroxyl (○) free radical concentrations over time. Time 0 corresponds to the intensity observed at baseline in the plasma, i.e., prior to the introduction of hypoxanthine and xanthine oxidase to the sample. This result is representative of experiments with six different plasma samples. Each sample had different initial levels of vitamins C and E. The endogenous levels of vitamins C and E in this sample were 63 and 48 μM, respectively. Introduction of additional hypoxanthine and xanthine oxidase at a time point beyond 125 min produced no change in the TO$^\bullet$ ESR signal intensity, whereas introduction at some time point where significant TO$^\bullet$ is observed (e.g., $\approx$ 45 minutes) produced a significant increase in $[TO^\bullet]_{ss}$. The poor signal-to-noise ratio in the TO$^\bullet$ signal intensity data will result in errors on the order of $\approx$ 10–25% in $[TO^\bullet]_{ss}$; the weaker the signal the more uncertainty. (It should be noted that under our experimental conditions, approximately 10 times more TO$^\bullet$ than $A^{\bullet-}$ is required to produce an ESR signal above the noise level. This is quite reasonable considering that TO$^\bullet$ has a broad 7-line spectrum whereas $A^{\bullet-}$ is a narrow doublet.)

longed exposure to oxidative stress $[TO^\bullet]_{SS}$ will decline.

Both ascorbate and tocopherol are able to donate two-electrons as antioxidants.[21–23] The observed levels of $A^{\bullet-}$ and TO$^\bullet$ represent steady-state concentrations of these species at a given time (Fig. 2). The formation of each radical will occur via

$$AH^- + R^\bullet \rightarrow A^{\bullet-} + RH$$

and

$$TOH + R^\bullet \rightarrow TO^\bullet + RH,$$

where R$^\bullet$ represents an oxidizing radical. Each antioxidant radical can decay by disproportionation

$$2A^{\bullet-} + H^+ \xrightarrow{k_{obs}^{(pH7.4)}=2\times10^5 M^{-1}s^{-1}} AH^- + \text{dehydroascorbate}^{21}$$

and

$$2TO^\bullet + H^+ \xrightarrow{k(\text{c-hexane})=3.5\times10^2 M^{-1}s^{-1}} TOH + \text{tocopherolquinone}.^{22}$$

Each radical could also decay by reacting directly with an oxidizing radical:

$$A^{\bullet-} + R^\bullet + H^+ \rightarrow RH + \text{dehydroascorbate}$$

and

$$TO^\bullet + R^\bullet + H^+ \rightarrow RH + \text{tocopherolquinone}.$$

No matter which route is taken to form dehydroascorbic or tocopherolquinone, each antioxidant serves as a two-electron reductant. Thus, in the absence of a system to recycle ascorbate, ascorbate will be consumed and $[A^{\bullet-}]_{SS}$ in the plasma will decline with time. In a parallel fashion, when ascorbate is depleted, TO$^\bullet$ can no longer be recycled to TOH, thus its steady-state concentration will also change with time, eventually approaching zero when reduced tocopherol is consumed.

Owing to a very low one-electron reduction potential for the ascorbate radical/ascorbate monoanion couple (Table 1), ascorbate serves as the terminal reductant in oxidizing free radical chain reactions.[23] Thus, nearly every oxidizing radical that arises in a biochemical or biological system (Table 1) would bring about the one-electron oxidation of ascorbate, forming the $A^{\bullet-}$. Ascorbate reacts rapidly with superoxide and hydroxyl radicals as well as with alkyl, peroxyl, and alkoxyl radicals,[21,24] thereby "repairing" these radicals and stopping the chain initiation and propagation reactions that begin with a free radical oxidative stress. Because the resonance-stabilized $A^{\bullet-}$

Table 1. One-Electron Reduction Potentials of Selected Radicals

Thermodynamic Couple	$E^{o\prime}$/mV	Reference #
$HO^{\bullet}$, H^+/H_2O	2310	31
$RO^{\bullet}$, H^+/ROH (aliphatic alkyoxyl radical)	1600	32
$ROO^{\bullet}$, H^+/ROOH (alkyl peroxyl radical)	1000	32
$O_2^{\bullet-}$, $2H^+/H_2O_2$	940	31
$GS^{\bullet}/GS^-$ (glutathione)	920	33
$PUFA^{\bullet}$, H^+/PUFA-H (polyunsaturated fatty acid, bis-allylic-H)	600	32
α-tocopheroxyl, H^+/α-tocopherol ($TO^{\bullet}$, H^+/TOH)	480	30
$Ascorbate^{\bullet-}$, H^+/Ascorbate monoanion	282	29

is relatively unreactive (compared to hydroxyl or lipid peroxyl radicals), its steady-state concentration serves as a marker for the degree of ongoing oxidative stress.[16,18,19]

Although the apparent synergistic relationship of vitamins C and E acting as antioxidants has been investigated in homogeneous solution,[13] micelles,[25,26] and liposomes,[27,28] this is the first direct ESR observation of the hieracheal relationship of ascorbate and tocopheroxyl free radicals in an *unsupplemented* biological fluid.

Furthermore, these ESR observations demonstrate that the predicted thermodynamic hierarchy of ascorbate, tocopherol, and their free radicals holds in a biological system containing endogeneous levels of these antioxidants. The reduction potential ($E^{O\prime}$) for the ascorbate radical/ascorbate couple is +282 mV (ref. 29) while that of the tocopheroxyl radical/tocopherol couple has been estimated to be +480 mV (ref. 30). Thus, for the repair of $TO^{\bullet}$ by ascorbate we can estimate that $\Delta E^{O\prime} \approx +200$ mV, a thermodynamically allowed process. Despite the fact that the concentrations of ascorbate, $A^{\bullet-}$, tocopherol, and $TO^{\bullet}$ in plasma are far removed from standard thermodynamic conditions (1.0 M), our results demonstrate that these reactions do indeed take place in biological fluids upon exposure to free radical oxidative stress.

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