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Protection by vitamin C of oxidant-induced loss of vitamin E in rat hepatocytes

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Studies with liposome model membranes demonstrate that vitamin C (ascorbic acid) can regenerate vitamin E (α-tocopherol) but in vivo experiments have yielded equivocal results concerning this recycling pathway. In vitro evaluation of vitamin C–vitamin E recycling in cell systems is lacking. We investigated the capacity of vitamin C to spare or regenerate vitamin E from loss during oxidative stress in primary rat hepatocytes. Livers from 3-month-old male Sprague-Dawley rats were perfused with collagenase and the hepatocytes incubated for 180 min in pH 7.4 Hank's balanced salt solution with 0.1% bovine serum albumin and the free radical generator 2,2′-azo-bis(2-amidinopropane) dihydrochloride (AAPH; 10–100 mM), a water soluble compound, or 2,2′-azo-bis-2,4-dimethylvaleronitrile (AMVN; 250–900 μmoles/L cell suspension), a lipid soluble compound, or a vehicle control. Hepatocyte α-tocopherol decreased by 75% in a dose-dependent fashion following AAPH treatment and was completely consumed following AMVN exposure. Loss of vitamin E was more rapid after treatment with AMVN than with AAPH (50% loss by 30 and 180 min with 900 μM AMVN and 10 mM AAPH, respectively) while declines were 30% or less under control conditions after 180 min. The addition of 2 to 6 mM vitamin C to the media after 15, 30, or 60 min prevented further loss of cellular vitamin E after AAPH or AMVN treatment. Vitamin C decreased the accumulation of α-tocopherolquinone by 35% following AMVN treatment, suggesting a recycling rather than an exclusive sparing action of vitamin C on vitamin E. Regardless of its specific mechanism of action, vitamin C can protect against the oxidant-induced loss of vitamin E in vitro. (J. Nutr. Biochem. 9:355–359, 1998) © *Elsevier Science Inc. 1998*

Keywords: vitamin C; vitamin E; free radicals; recycling; hepatocytes; oxidative stress

Introduction

Vitamin E (α-tocopherol) is a fat soluble vitamin located principally within the lipid compartment of cellular and subcellular membranes. It functions predominantly as an antioxidant, responsible for terminating free radical chain reactions that result from the oxidation of polyunsaturated fatty acids.[1] After α-tocopherol donates reducing potential to quench a free radical, it becomes a resonance-stabilized tocopheroxyl radical, which, after quenching a second radical, can decompose to α-tocopherolquinone and related compounds that lack antioxidant activity. Although α-tocopherol is very effective at breaking lipid peroxidation chain reactions, it exists at a ratio of approximately 1:2000 relative to target molecules for peroxidation.[2] This may be partially explained by the rate at which lipid peroxyl radicals are formed and the efficiency with which α-tocopherol can quench them, but it also has been proposed that vitamin C (ascorbic acid) or other cytosolic compounds might regenerate α-tocopherol from its tocopheroxyl radical or other intermediate form and thereby restore its free radical scavenging capacity.[3,4]

In vitro studies with model membrane systems suggest

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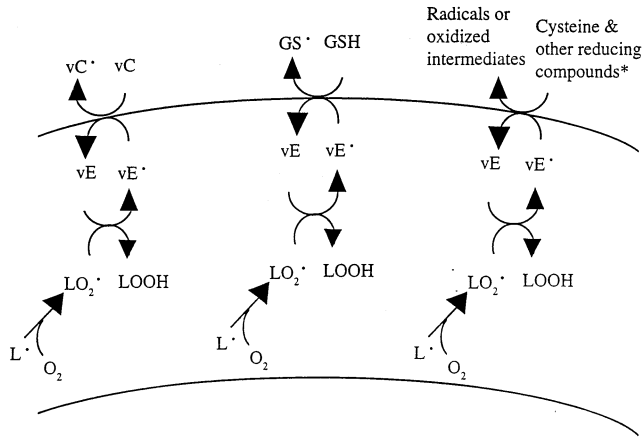

Figure 1 Hypothetical scheme of vitamin E recycling by vitamin C and other compounds.

that vitamin C, and to a lesser degree glutathione (GSH), can regenerate intermediate oxidized metabolites of α-tocopherol during lipid peroxidation (*Figure 1*). Böhm et al.[5] have reported using pulse radiolysis and laser flash photolysis in methanol and hexane solutions that carotenoids enhance vitamin C recycling of vitamin E by transferring the reducing potential from ascorbic acid to α-tocopherol. Using electron spin resonance (ESR), Tsuchiya et al.[6] demonstrated that either ascorbate or GSH could quench phenoxyl radicals located in micellar and liposomal membranes. By monitoring the formation of the ascorbyl radical, Stoyanovsky et al.[7] also provided ESR evidence that endogenous ascorbate can regenerate the phenoxyl radical of α-tocopherol. On exposure to ultraviolet radiation, α-tocopherol in rat retinal homogenates did not decline until after the ascorbyl radical signal was lost. Chan et al.[8] demonstrated that the addition of ascorbate to platelets exposed to arachidonic acid resulted in a restoration of α-tocopherol that had been lost during oxidation. The addition of GSH had a similar but less dramatic restorative effect on α-tocopherol.

The few in vivo studies that examine vitamin C–vitamin E recycling have presented equivocal results, although definitive data on this interaction may be particularly difficult with such an approach due to many potential confounding factors. Chen et al.[9] fed weanling rats varying levels of vitamin E and vitamin C and concluded that vitamin C may spare the metabolism of vitamin E. Rats that were fed diets deficient in vitamin E but supplemented with vitamin C had plasma vitamin E levels 75% greater than rats fed the same diet without supplemental vitamin C. Burton et al.[10] investigated the interaction by feeding guinea pigs deuterium-labeled α-tocopherol and monitoring its loss from tissues when the animals were fed different levels of vitamin C. The loss of deuterated α-tocopherol from tissue was similar at all levels of vitamin C, leading the authors to conclude that the sparing effect of vitamin C on vitamin E documented in vitro may be of negligible importance in vivo. In a similar study, Jacob et al.[11] fed healthy women deuterated α-tocopherol and then placed them on differing levels of ascorbate intake. Although the loss of deuterated α-tocopherol from plasma and platelets was similar between the different ascorbate groups, the loss of deuterated α-tocopherol from buccal cells was slower in the high vitamin C group than in the low vitamin C group.

In vitro evaluation of vitamin C–vitamin E recycling in cell systems is lacking. We investigated the capacity of vitamin C to spare or regenerate vitamin E from loss during oxidative stress in primary rat hepatocytes.

Methods and materials

Materials

2,2′-Azo-bis-(2-amidinopropane) dihydrochloride (AAPH) and 2,2′-azo-bis-2,4-dimethylvaleronitrile (AMVN) were obtained from Wako Chemicals (Richmond, VA). All other chemicals were reagent grade and were obtained from Sigma Chemical Co. (St. Louis, MO). All solvents were of high performance liquid chromatography (HPLC) grade and used without further purification.

Preparation of isolated hepatocytes

Male Sprague-Dawley rats aged 3- to 6-months-old were allowed food and water *ad libitum*. Rats were anesthetized with methoxyflurane and hepatocytes were isolated by collagenase perfusion.[12] Briefly, the liver was perfused via the portal vein with a Hank's balanced salt solution (HBSS) containing ethylene glycol-bis-(β-aminoethylether)-N,N,N′,N′-tetraacetic acid followed by a collagenase perfusion. The liver was removed and hepatocytes washed and resuspended in a Hank's buffer containing 0.1% bovine serum albumin. This procedure yielded 70 to 100 $\times$ 10^6 hepatocytes/liver that were greater than 80% viable as measured by trypan blue exclusion.[12]

Treatment and incubation of hepatocytes

One milliliter aliquots of hepatocyte suspension (2×10^6 cells/ml) were treated with either AMVN, AAPH, or control vehicle (HBSS or ethanol, 0.75%) and placed in a water bath at 37°C. After either 15, 30, or 60 minutes, ascorbic acid was added to the cell suspension and the incubation allowed to continue for up to 180 minutes. Aliquots were removed at indicated time points and assayed in duplicate. Each 1.0 ml sample contained approximately 1 mg protein.

Vitamin E analysis

Hepatocyte α-tocopherol and α-tocopherolquinone were determined by a modification of the method developed by Vatassery and Smith,[13] which is optimized to limit the loss and formation during the assay of α-tocopherol and α-tocopherolquinone, respectively. Briefly, 1 ml aliquots of hepatocytes were mixed with 0.2 ml 15% ascorbic acid, 2 ml ethanol containing 0.025% butylated hydroxytoluene, and 1 ml 10% potassium hydroxide. Samples were incubated at 45°C for 30 minutes. α-Tocopherol and α-tocopherolquinone were extracted with hexane and measured by reverse-phase HPLC with fluorescence and ultraviolet (UV) detectors in series. α-Tocopherol was monitored at excitation and emission wavelengths of 285 and 330 nm, respectively. α-Tocopherolquinone was measured by UV detection at 265 nm. Internal standards of tocol and cholecalciferol were used to measure α-tocopherol and α-tocopherolquinone, respectively.

Vitamin C analysis

Hepatocyte ascorbate was determined by reverse-phase HPLC using a modification of the method developed by Mitton and

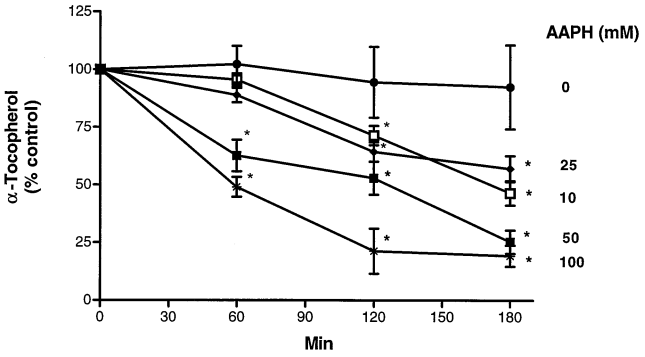

Figure 2 Effect of AAPH concentration on hepatocyte α-tocopherol. *Significantly different from control. $P < 0.05$.

Trevithick.[14] Briefly, 1 ml aliquots of hepatocytes were centrifuged, the supernatant was removed, and the pellet was resuspended in 1 ml 2% metaphosphoric acid containing 0.54 mM ethylenediaminetetraacetic acid. The sample was sonicated and centrifuged, and 20 μl of supernatant was measured at 0.5V using electrochemical detection.

Statistical analysis

Data points in the figures represent the mean ± standard deviation of duplicate measurements from at least two experiments. Duplicate measurements in each experiment were within 5% of each other. A two-tailed Student's *t*-test was used to determine significant differences between treated and nontreated groups. Means were considered different at *P*-values of less than 0.05. Because initial hepatocyte α-tocopherol values can vary substantially from one animal to another, results are expressed as percent of baseline control. Initial hepatocyte α-tocopherol values ranged from 180 to 350 pmol/10^6 cells.

Results

When hepatocytes were incubated with AAPH (10–100 mM), α-tocopherol decreased in a dose-dependent fashion over time (*Figure 2*). Incubation of hepatocytes with 100 mM AAPH for 180 minutes resulted in a 75% loss of α-tocopherol, whereas cells treated with a vehicle control (HBSS) lost 10% or less of their initial vitamin E content. Hepatocytes treated with AAPH lost their ascorbate content in a similar, yet slightly faster, manner. Treatment with 100 mM AAPH led to a 90% loss of hepatocyte ascorbate after 180 minutes (*Figure 3*). When 5 mM ascorbic acid was added to hepatocyte suspensions 60 minutes after treatment

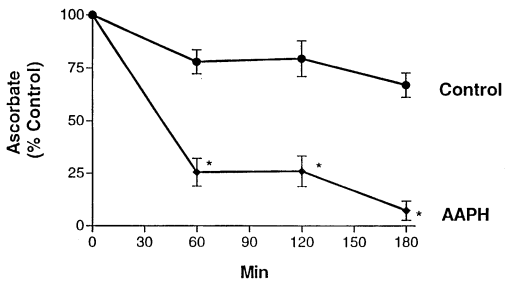

Figure 3 Effect of 100 mM AAPH on hepatocyte ascorbate (Asc). *Significantly different from control. $P < 0.05$.

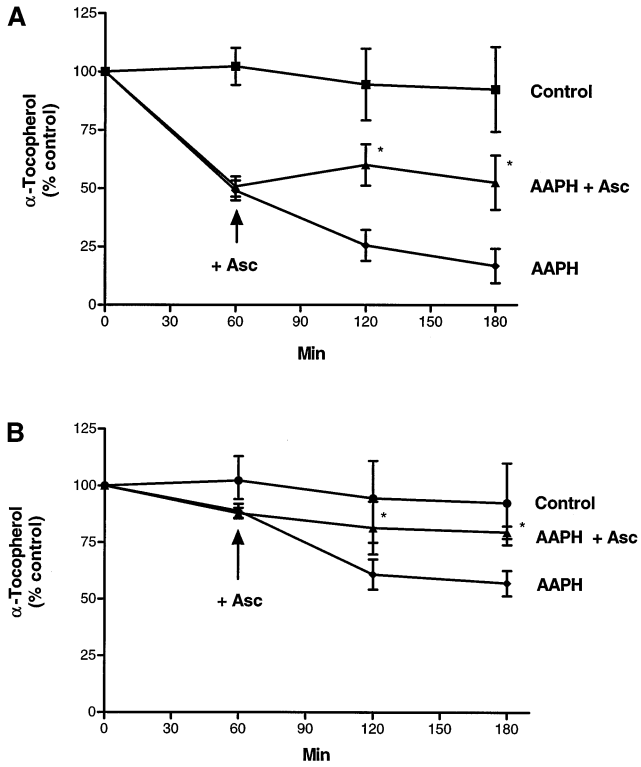

Figure 4 (*Figure 4A*) Effect of 5 mM ascorbate added at 60 minutes on α-tocopherol loss in hepatocytes treated with 100 mM AAPH. *Significantly different from group receiving AAPH. $P < 0.05$. (*Figure 4B*) Effect of 6 mM ascorbate added at 60 minutes on α-tocopherol loss in hepatocytes treated with 25 mM AAPH. *Significantly different from group receiving AAPH. $P < 0.05$.

with 100 mM AAPH, further loss of hepatocyte α-tocopherol was prevented during the incubation period (*Figure 4A*). Similar results were obtained with 6 mM ascorbic acid and 25 mM AAPH (*Figure 4B*).

Hepatocytes were incubated with AMVN (250–900 μmol/L cell suspension) for 180 minutes and α-tocopherol decreased in a dose-dependent fashion, with a complete loss of vitamin E resulting from the highest dose treatment (*Figure 5*). Control cells in the AMVN experiments lost a greater amount of α-tocopherol than controls in the AAPH experiments, 25% and 10%, respectively. This situation appears to be due to the use of ethanol as the vehicle control for the added AMVN. Recently, Kurose et al.[15] demon-

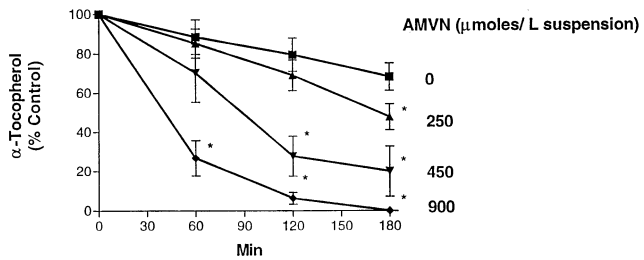

Figure 5 Effect of AMVN (μmoles/L suspension) concentration on hepatocyte α-tocopherol. *Significantly different from control. $P < 0.05$.

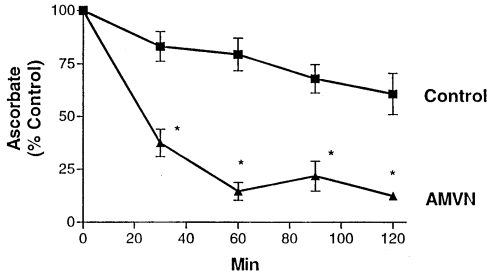

Figure 6 Effect of 500 μmoles/L cell suspension AMVN on hepatocyte ascorbate levels. *Significantly different from control. $P < 0.05$.

strated that ethanol can have an oxidative impact on hepatocytes. In a separate experiment, 1.0 ml aliquots of hepatocyte suspension incubated with 1%, 5%, or 10% ethanol lost 23, 42, and 76%, respectively, of their initial α-tocopherol levels after 180 minutes. Hepatocytes incubated with 200 μmoles/L cell suspension AMVN in 1% ethanol lost 84% of their initial α-tocopherol after 180 minutes. Treatment with 500 μmoles/L cell suspension AMVN led to 85% loss of hepatocyte ascorbate after 120 minutes (*Figure 6*). The addition of 6 mM ascorbic acid after 15 minutes to hepatocytes treated with 500 μmoles/L cell suspension AMVN diminished further loss of α-tocopherol over the course of the incubation (*Figure 7A*). Cells treated with AMVN and vitamin C lost 53% of their initial α-tocopherol compared with a 93% loss in AMVN-treated hepatocytes. The formation of α-tocopherolquinone in AMVN-treated hepatocytes was also diminished with the addition of vitamin C (*Figure 7B*).

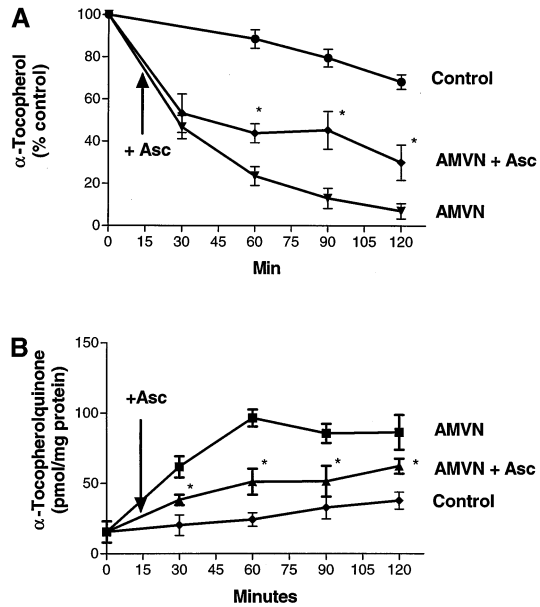

Figure 7 (*Figure 7A*) Effect of 5.5 mM ascorbate added at 15 minutes on α-tocopherol loss in hepatocytes treated with 500 μmoles/L cell suspension AMVN. *Significantly different from group receiving AMVN. $P < 0.05$. (*Figure 7B*) Effect of 5.5 mM ascorbate added at 15 minutes on α-tocopherolquinone formation in hepatocytes treated with 500 μmoles/L cells suspension AMVN. *Significantly different from group receiving AMVN. $P < 0.05$.

Discussion

The ability of vitamin C to recycle vitamin E has been the focus of research for several years, yet interpretation of the available data is varied. For example, Gey[16] stated that "Regeneration of the lipid-soluble vitamin E from its radical by vitamin C . . . is very well documented and may be of major physiological importance." In contrast, Draper[17] stated that "Despite strong evidence that vitamin C is capable of regenerating vitamin E . . . under laboratory conditions, evidence for this reaction from animal experiments is uniformly negative." Although definitive in vivo evidence demonstrating a recycling capacity of vitamin E by vitamin C is lacking, studies with liposomes and other membrane systems suggest such an interaction.[6,18–20] However, several design and methodologic problems make it difficult to distinguish between a sparing effect of vitamin C, whereby ascorbic acid provides an additive effect by quenching reactive oxygen species and prolonging the time before α-tocopherol is utilized and consumed, and a recycling action with ascorbate converting the tocopheroxyl radical (or other oxidized tocopherol intermediate) to α-tocopherol by providing reducing potential.

The demonstration that vitamin C prevents the AAPH-induced loss of hepatocyte vitamin E content (*Figure 4*) could reflect a simple sparing action because ascorbate might interact directly with peroxyl radicals generated in the cytosol by the thermal decomposition of AAPH. Vitamin C declined in hepatocytes treated with AAPH or AMVN; in both cases the decline of ascorbate was greater and preceded the decline of vitamin E. Although the decline of vitamin C induced by the water soluble AAPH could be anticipated, ascorbate would appear less able to participate in a direct interaction to quench AMVN-generated radicals due to their compartmentalization in lipids. Consistent with this suggestion, Niki et al.[19] reported that ascorbate could not slow oxygen uptake from AMVN-treated liposomes unless α-tocopherol was present.

In hepatocytes, vitamin C was effective in preventing both the AMVN-induced decrease in α-tocopherol (*Figure 7A*) and the accumulation of its principal oxidized metabolite α-tocopherolquinone (*Figure 7B*). The decreased rate of formation of α-tocopherolquinone suggests that the degradative pathway of α-tocopherol was affected by vitamin C through a direct nutrient–nutrient interaction. Ascorbate may also increase cell viability and, thus, indirectly preserve vitamin E. Cell viability was assessed here only after isolation of the hepatocytes, not throughout the experiments. The inhibition of α-tocopherolquinone production by ascorbate was less than proportionate to the preservation of α-tocopherol. This effect was anticipated because the oxidation of α-tocopherol was initiated after the oxidant stress and 15 to 60 minutes prior to the addition of ascorbate. Added ascorbate cannot recycle oxidized tocopherol metabolites (e.g., the tocopherol cation) past the α-tocopheroxyl radical step. Further clarification of this interaction may be achieved through experiments that assess simultaneously oxidant-induced changes in ascorbic acid and α-tocopherol and their respective oxidation products.

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