Effects of glutathione on the α-tocopherol-dependent inhibition of nuclear lipid peroxidation

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Abstract Endogenous α-tocopherol levels in isolated rat liver nuclei were determined to be 0.045 mol% (mol α-tocopherol per mol phospholipid × 100). This value corresponds to 970 polyunsaturated fatty acid (PUFA) moieties to one molecule of α-tocopherol in the nuclear membrane. Isolated nuclei, when incubated with various concentrations of exogenous α-tocopherol, took up only a small percent of initial levels of α-tocopherol present in the incubation media. Exogenous α-tocopherol, when incorporated in isolated nuclei above a threshold value of 0.085 mol%, effectively inhibited NADPH-induced lipid peroxidation. The addition of 1 mM glutathione lowered the threshold levels of α-tocopherol needed to inhibit lipid peroxidation to about 0.040 mol%. We suggest the data indicate a glutathione-dependent enhancement of the ability of α-tocopherol to inhibit nuclear lipid peroxidation. —**Tirmenstein, M. A., and D. J. Reed.** Effects of glutathione on the α-tocopherol-dependent inhibition of nuclear lipid peroxidation. *J. Lipid Res.* 1989. **30:** 959–965.

Supplementary key words polyunsaturated fatty acids • rat liver nuclei • vitamin E

Vitamin E is believed to function as an important cellular antioxidant. α-Tocopherol (α-TH), a component of vitamin E, inhibits in vitro lipid peroxidation in a variety of membrane systems (1–3). Evidence suggests that α-TH exerts its antioxidant activity by scavenging free radicals generated in lipid peroxidation (4). Previous studies conducted in this laboratory have shown that α-TH incorporated in soybean phosphatidylcholine liposomes inhibited iron-induced lipid peroxidation. Protection against peroxidation occurred only when α-TH levels were incorporated into liposomes above a certain critical level (5). Similar results were reported by Fukazawa et al. (6) using egg lecithin liposomes. α-Tocopherol, when incorporated in liposomes above a threshold value of 2 nmol/mmol egg lecithin, completely inhibited lipid peroxidation whereas subthreshold concentrations of α-TH failed to protect against peroxidation.

In addition to the findings with vitamin E, several studies have demonstrated that glutathione (GSH) can inhibit microsomal lipid peroxidation (7–9). The addition of GSH produced a lag period prior to the onset of microsomal lipid peroxidation in these studies. Following the cessation of this lag period, lipid peroxidation ensued at a rate characteristic of incubations lacking GSH (7). Although the mechanism involved in this GSH-dependent inhibition remains in doubt, several lines of evidence suggest that a microsomal protein is required for protection by GSH (7, 8).

Much the same results were obtained from experiments conducted in this laboratory with isolated rat liver nuclei and GSH (10). Glutathione protected isolated nuclei from lipid peroxidation by inducing a lag period prior to peroxidation. Evidence obtained from these experiments suggest that a nuclear membrane protein is involved in this GSH-dependent protection and that this protein possesses a GSH-dependent peroxidase activity (10).

The following study examines the effects of GSH on the ability of α-TH to inhibit nuclear lipid peroxidation. Nuclear α-TH levels were augmented by preincubating isolated nuclei with exogenous α-TH. Following these preincubations, the levels of α-TH required to inhibit lipid peroxidation were determined. The data indicate that GSH enhances the ability of α-TH to protect against lipid peroxidation.

EXPERIMENTAL PROCEDURES

Chemicals

Glutathione, NADPH (type I) and ADP were purchased from Sigma Chemical Co. (St. Louis, MO). α-Tocopherol and γ-tocopherol were obtained from Eastman Kodak Co. (Rochester, NY).

Abbreviations: α-TH, α-tocopherol; GSH, glutathione; HPLC, high performance liquid chromatography; MDA, malondialdehyde; PUFA, polyunsaturated fatty acids.

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Animals

Male Sprague-Dawley rats (Simonsen Labs., Gilroy, CA) (250–300 g) were used throughout the course of this study. Animals had free access to Purina Rat Chow and water.

Isolation of rat liver nuclei and preincubation with α-tocopherol

Rats were anesthetized with ether, then decapitated. Livers were immediately removed and placed on ice. Nuclei were isolated from rat liver according to Blobel and Potter (11) with slight modifications for large scale preparations utilizing a Beckman SW 28 rotor. After isolation, nuclear pellets were resuspended in 10 ml of 100 mM NaCl buffer TM (50 mM Tris, 5 mM $MgCl_2$, pH 7.5) at a concentration of about 2 mg protein per ml. Various concentrations of α-TH were dissolved in absolute ethanol and added to nuclear suspensions so that the final concentration of ethanol was 1% (v/v). Nuclear suspensions containing α-TH were incubated at 37°C for 15 min with swirling. Nuclei were pelleted after incubation by centrifuging at 4000 *g* for 10 min at 4°C. Nuclei were washed with 20 ml of 100 mM NaCl buffer TM to remove unincorporated α-TH.

Peroxidation of isolated nuclei

Nuclear pellets containing added α-TH were resuspended in 100 mM NaCl buffer TM at 37°C. The peroxidation system consisted of 1 mM NADPH, 1.7 mM ADP, 0.11 mM EDTA, and 0.1 mM $FeCl_3$ (12). Peroxidation was initiated at time zero by the addition of the ADP, EDTA, and $FeCl_3$ solution.

Biochemical determinations

α-Tocopherol levels were measured by reverse phase HPLC with fluorescence detection as described by Fariss, Pascoe, and Reed (13) with slight modifications. γ-Tocopherol, an internal standard, was added to nuclear suspensions according to the procedures of Fariss et al. (13) prior to extraction with hexane (14). Hexane extracts were evaporated under nitrogen, and the residue was dissolved in methanol. γ-Tocopherol and α-TH were eluted isocratically from a Spherisorb ODS-II column (4.6 × 260 mm) (Alltech, Waukegan, IL) with 95% methanol in water (v/v) at a flow rate of 1.7 ml/min.

Proteins were determined according to the procedures of Lowry et al. (15) as modified by Peterson (16). Phosphorus levels were measured using the procedures of Fiske and SubbaRow (17) as modified by Bartlett (18).

Malondialdehyde (MDA) levels were determined by using the thiobarbituric acid assay as developed by Wills (19) with the following modifications. Aliquots (0.25 ml) of nuclear suspensions were added to 0.5 ml 10% (w/v) trichloroacetic acid. The resulting mixture was cooled to 4°C and then centrifuged for 2 min at 15,000 *g*. Supernatants were decanted off and added to 1.0 ml of 0.56% (w/v) thiobarbituric acid. Samples were heated at 95°C for 20 min and then cooled to room temperature. Absorbance values were measured at 532 nm.

RESULTS

Endogenous levels of α-TH were measured in isolated rat liver nuclei. A value of 0.039 ± 0.009 nmol α-TH per mg protein (mean ± standard error, n = 9) was obtained. Since a certain amount of autoxidation of α-TH may occur during nuclear isolation, this value represents a minimum value for endogenous levels of nuclear α-TH. However, when butylated hydroxyanisole was included in the isolation media to reduce the autoxidation of α-TH, the amount of α-TH detected in nuclei was not significantly increased (data not shown). A value of 0.045 mol % α-TH (mol α-TH per mol phospholipid × 100) was calculated from a conversion factor of 86.7 nmol phospholipid per mg protein. Protein values were determined instead of phosphate levels because of the ease and increased sensitivity of the protein assay. Previous research in this laboratory has shown that 43.5% of the total moles of fatty acids in isolated rat liver nuclei is polyunsaturated (10). Based on these data, about 970 nmol of PUFA is present per nmol of α-TH in isolated rat liver nuclei.

A series of incubations was performed to augment the levels of α-TH incorporated in isolated nuclei. Nuclear suspensions (2 mg protein per ml) were exposed to various concentrations of α-TH, then pelleted and washed. **Fig. 1** shows the percent of initial levels of α-TH added to incubations that remained associated with nuclei following these incubations and washings. Only a small percent of the initial levels of α-TH was incorporated into isolated nuclei.

Experiments utilizing nuclei supplemented with α-TH were conducted to assess the ability of exogenous α-TH to protect against lipid peroxidation (as measured by MDA formation). Time points for α-TH levels and the corresponding MDA formation following exposure of supplemented nuclei to peroxidation are reported in **Table 1**. Nuclei supplemented with α-TH exhibited much lower levels of MDA formation during the 60-min incubation period than those measured in control nuclei. Little loss of α-TH was detected in nuclei supplemented with high α-TH levels.

The effects of GSH on the ability of exogenous α-TH to protect against lipid peroxidation are shown in **Table 2.** The addition of GSH inhibited the loss of α-TH and

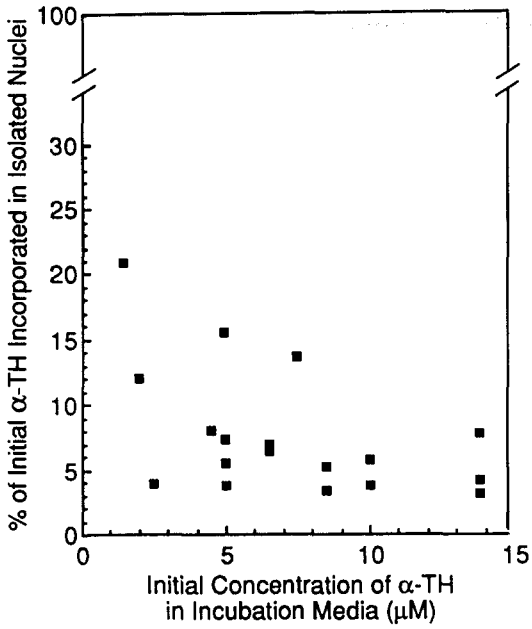

Fig. 1. The uptake of α-TH by isolated rat liver nuclei. Isolated nucle suspensions (2 mg protein per ml) were preincubated with various concentrations of α-TH for 15 min at 37°C with swirling. The final concentration of ethanol present in these suspensions was 1% (v/v). Following preincubations, nuclei were pelleted at 4000 *g* for 10 min at 4°C. Nuclei were then washed to remove unincorporated α-TH and assayed for α-TH as described in the Experimental Procedures section. All values plotted in Fig. 1 were corrected for endogenous levels of α-TH.

the formation of MDA. Nuclei supplemented with 0.107 mol % α-TH exhibited a 30-min lag period prior to the onset of lipid peroxidation. Nuclei containing similar levels of exogenous α-TH as well as GSH displayed only low levels of MDA formation during the incubation period and little loss of α-TH levels.

The results of several time course experiments (as shown in Tables 1 and 2) were compiled to determine what levels of α-TH are required to inhibit lipid peroxidation. Endogenous levels of α-TH in nuclei were altered by preincubating nuclear suspensions with varying amounts of exogenous α-TH. Nuclei were then washed and exposed to peroxidizing conditions as previously described. Zero time points were not included in these plots. The pooled results from experiments in which no GSH was added are displayed in **Fig. 2A.** Above 0.085 mol percent α-TH, little to no lipid peroxidation was detected. However, below this value, progressively higher values of MDA were formed. The sharp inflection point or threshold value at which α-TH becomes ineffective in inhibiting lipid peroxidation is clearly demonstrated by examining the percent of data points yielding values above 1 nmol MDA per mg protein. Above 0.100 mol % α-TH, none of the data points were above 1 nmol MDA per mg protein; below 0.079 mol percent α-TH, 100% of the data points were above 1 nmol MDA per mg protein.

Fig. 2B shows the results of experiments in which 1 mM GSH was included in the incubation media. Incubation and peroxidation conditions were the same as those shown in Fig. 2A with the exception of the addition of GSH. The threshold value determined from these data was about 0.04 mol % α-TH. In these experiments, the addition of GSH increased the effectiveness of α-TH by lowering the levels of α-TH required for complete protection of nuclei against lipid peroxidation. Above 0.060 mol % α-TH, none of the data points were above 1 nmol MDA per mg protein; below 0.019 mol %, 100% of the data points were above 1 nmol per mg protein. The levels of PUFA protected by α-TH are reported in **Table 3.** Results indicate that α-TH in the presence of GSH can protect about twice the number of molecules of PUFA as it can without added GSH.

DISCUSSION

Endogenous α-TH levels in isolated rat liver nuclei were determined to be 0.045 mol %. Calculations estimate that this level correlates with a ratio of one molecule

TABLE 1. Time course of α-TH depletion and induced lipid peroxidation in isolated rat liver nuclei

	α-TH Supplemented Nuclei[a,b]		Control Nuclei[b]	
Time	α-TH (mol %)	nmol MDA/mg Protein	α-TH (mol %)	nmol MDA/mg Protein
min				
0	0.290	0.05	0.045	0.04
15	0.221	0.19	N.D.[c]	3.52
30	0.279	0.21	N.D.	6.50
45	0.244	0.27	N.D.	8.29
60	0.265	0.28	N.D.	9.74

[a]Nuclei were supplemented with α-TH according to the methods in the Experimental Procedures section.
[b]One experiment typical of five separate experiments.
[c]Not detectable.

TABLE 2. The effects of GSH on the time course of α-TH depletion and induced lipid peroxidation in isolated rat liver nuclei supplemented with α-TH

	- GSH[a]		+ GSH[a,b]	
Time	α-TH (mol %)	nmol MDA/mg Protein	α-TH (mol %)	nmol MDA/mg Protein
min				
0	0.107	0.08	0.099	0.17
15	0.118	0.37	0.082	0.23
30	0.087	1.15	0.078	0.22
45	0.034	3.72	0.077	0.23
60	N.D.[c]	6.51	0.099	0.28

[a]One experiment typical of four separate experiments.
[b]One mM GSH.
[c]Not detectable.

of α-TH to 970 PUFA moieties in the nuclear membrane. We were unable to find other values in the literature for nuclear levels of α-TH in isolated rat liver nuclei. However, studies conducted with other rat liver subcellular organelles have reported values of 3313 and 2100 for the ratio of PUFA moieties to α-TH in microsomes (20) and mitochondria (21), respectively.

The present experiment indicates that isolated rat liver nuclei incorporate exogenous α-TH, but that the percent of the initial α-TH incorporated in nuclei after washing is relatively low. Once taken up by membranes, the exogenous α-TH effectively inhibited NADPH-induced lipid peroxidation. Analogous studies assessing the ability of exogenous α-TH to protect rat liver microsomes were performed by Cadenas et al. (22) and Ohki, Takamura, and Nozawa (3). Ohki et al. (3) demonstrated that exogenous α-TH inhibited lipid peroxidation even at the lowest concentration incorporated in washed microsomes.

In studies conducted by Cadenas et al. (22), virtually all added α-TH remained associated with microsomes following centrifugation. The exogenous α-TH inhibited lipid peroxidation but did so 50 times less effectively than endogenous α-TH. These researchers concluded that α-TH must be present at certain specific sites if it is to efficiently protect membranes against peroxidation. It is important to note that the microsomes supplemented with α-TH in this study were not washed prior to exposure to the peroxidation system. Unlike the preparations used in the present study and by Ohki et al. (3), washing may remove α-TH that is not fully incorporated or bound in the membrane and is therefore less effective in inhibiting lipid peroxidation. Support for this hypothesis is provided by experiments utilizing a model peroxidation system consisting of microsomal structural proteins in lipid micelles (1). Data from this research suggest that vitamin E bound to structural proteins inhibited lipid peroxidation while unbound vitamin E did not.

The present study supports the validity of the α-TH threshold effect in predicting the ability of α-TH to inhibit lipid peroxidation. Membrane α-TH levels above about 0.085 mol % inhibited nuclear lipid peroxidation, but values below this level were associated with high amounts of peroxidation. The reason for this threshold effect is unknown but may relate to the autocatalytic nature of lipid peroxidation. The initiation of lipid peroxidation starts a free radical chain reaction producing reactive chemical species such as lipid hydroperoxides which can then initiate further lipid peroxidation. The length of these chain reactions ultimately determines how many lipid peroxidation chain branching initiation sites are produced (23). α-TH inhibits lipid peroxidation by terminating free radical chain reactions. If enough α-TH is present in the membrane, the chain reaction is rapidly terminated and few chain branching initiation sites are produced. In addition, little α-TH is oxidized under these circumstances. If, however, α-TH levels are not sufficient to quickly terminate these chain reactions, large amounts of chain branching initiation sites are produced which subsequently consume existing α-TH supplies and peroxidize PUFAs. The threshold effect for α-TH has been shown to occur in experiments using liposomes (5, 6) but the present study is the first demonstration that the threshold effect also occurs in native membrane systems.

The addition of GSH to incubations was found to markedly lower the amount of α-TH required to inhibit NADPH-induced lipid peroxidation. α-TH threshold levels decreased from 0.085 mol % to 0.040 mol % following the addition of 1 mM GSH. Previous studies in this laboratory have shown that a nuclear protein is responsible for the GSH-dependent inhibition of nuclear peroxidation (10). Two mechanisms may account for this GSH-dependent enhancement of the capacity of α-TH to inhibit lipid peroxidation. The enzymes glutathione transferase (24) and glutathione peroxidase (25) catalyze

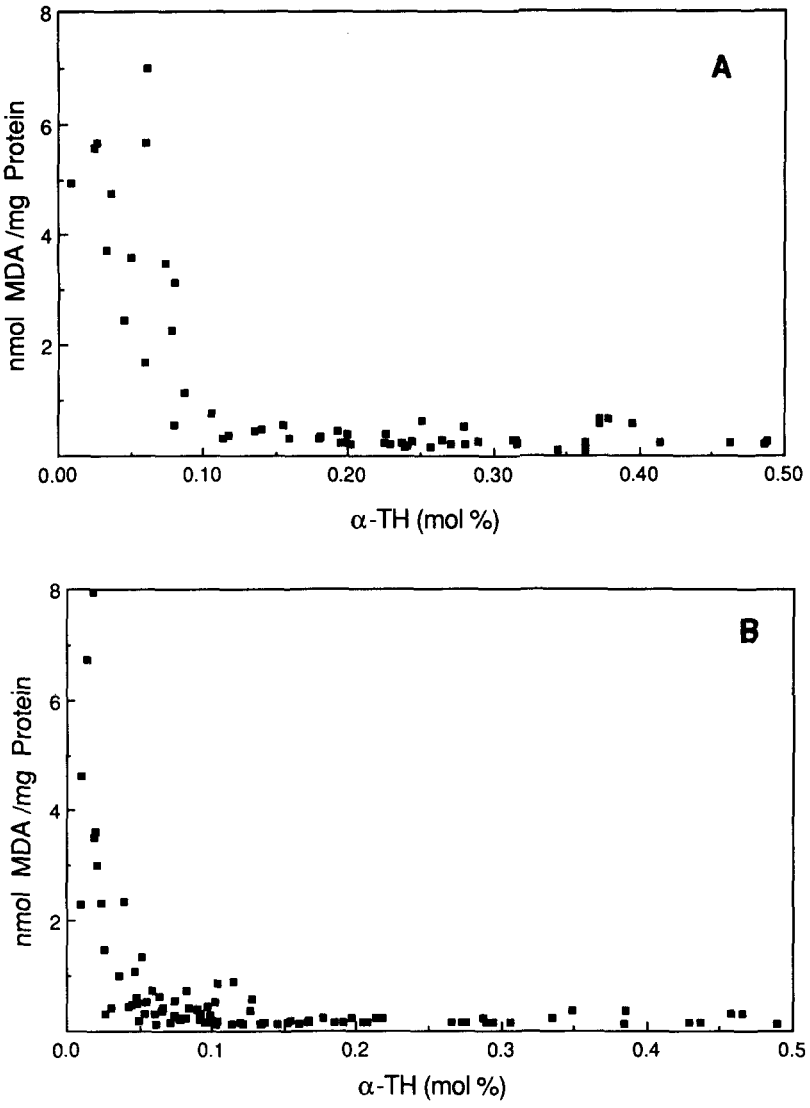

Fig. 2. **A.** The levels of α-TH required to inhibit NADPH-induced nuclear peroxidation. This figure is a compilation of a series of time course experiments (i.e., Table 1) in which α-TH-supplemented nuclei were exposed to peroxidation. The procedures followed were the same for each experiment and are identical to Table 1 except that the levels of α-TH preincubated with nuclei were different in each experiment. The levels of α-TH given in the abscissa reflect both endogenous and exogenous α-TH levels. Zero time points were not included in this plot. The observed inflection point was seen in four separate experiments. B. The effects of GSH on the levels of α-TH required to inhibit NADPH-induced nuclear peroxidation. This figure is a compilation of a series of time course experiments (i.e., Table 2) in which α-TH supplemented nuclei were exposed to peroxidation in the presence of 1 mM GSH. The procedures followed were the same for each experiment and are identical to Table 2 except that the levels of α-TH preincubated with nuclei were different in each experiment. All incubations contained 1 mM GSH. The levels of α-TH given in the abscissa reflect both endogenous and exogenous nuclear α-TH levels. Zero time points were not included in this plot. The observed inflection point was seen in four separate experiments.

the GSH-dependent reduction of lipid hydroperoxides to lipid alcohols. Studies indicate that these enzyme activities can be associated with cellular membranes (26, 27). Since lipid hydroperoxides can initiate lipid peroxidation, the reduction of these compounds can contribute to the inhibition of peroxidation. The reduction of lipid hydroperoxides should decrease the number of chain branching initiation sites and thereby increase the effec-

TABLE 3. Levels of α-TH required to protect polyunsaturated fatty acids (PUFA) from lipid peroxidation

	nmol PUFA/nmol α-TH
Isolated nuclei	510[a]
Isolated nuclei + 1 mM GSH	1,100[a]

[a]Based on 43.5% of total moles of fatty acids being polyunsaturated (10).

tiveness of existing levels of α-TH. Shlyapnikov and Miller (28), using kinetic models, predicted that agents that reduce lipid hydroperoxides act to enhance the ability of antioxidants such as α-TH to inhibit lipid peroxidation.

Another mechanism which may account for the observed interaction between α-TH and GSH was proposed by Reddy et al. (9). They reported that the GSH-dependent inhibition of microsomal lipid peroxidation occurred in microsomal preparations from control rats but not in microsomes prepared from vitamin E-deficient rats. On the basis of these data, the existence of a microsomal protein that utilizes GSH to regenerate vitamin E was postulated.

In the nuclear peroxidation system, the addition of GSH reduced the threshold levels of α-TH required to protect against peroxidation. If GSH does regenerate α-TH levels through the actions of a nuclear protein, the rate of lipid peroxidation and α-TH consumption may be reduced but the absolute amount of α-TH required for protection should remain unchanged. Although the data presented in this report do not discount either hypothesis, the evidence seems to suggest that GSH functions independently of α-TH to inhibit lipid peroxidation.

The levels of α-TH needed to protect against peroxidation in nuclear membranes were about five times less than those needed to inhibit peroxidation in liposomal membranes (5). In a study conducted by Liebler, Kling, and Reed (5), liposomes prepared from egg phosphatidylcholine possessed an α-TH threshold value of about 100 nmol PUFA per nmol α-TH. An increased susceptibility of liposomal membranes as opposed to native membranes was also noted by Fukuzawa et al. (2). The explanation of this effect may relate to differences in membrane structure. Biological membranes contain such compounds as cholesterol which has been shown to suppress lipid peroxidation in liposomal membranes (29). Although cholesterol may be oxidized (30), it is more resistant than PUFAs to oxidation (30). Also, other membrane components such as proteins or other antioxidants may protect against lipid peroxidation.

In the presence of 1 mM GSH, one molecule of α-TH protected 1,100 PUFA moieties; while in the absence of GSH, α-TH protected only 510 PUFA moieties. It is interesting to note that a similar threshold value of α-TH was reported by Bieri and Poukka (31). In their experiments, a value of one molecule of α-TH to 1,100 PUFA moieties in rat erythrocytes was required to inhibit red cell hemolysis. Endogenous levels of α-TH in rat liver nuclei were slightly higher than the threshold levels of α-TH needed to protect nuclei in the presence of 1 mM GSH but below threshold values determined in the absence of GSH. Since the concentration of GSH utilized in this study is similar to those found intracellularly (32), this report suggests that GSH may be important in extending the ability of α-TH to inhibit lipid peroxidation in vivo.

We wish to thank Lynne Rogers for her excellent technical assistance in preparing this manuscript. This work was supported in part by NIH grant ES01978 and grant CH-109 from the American Cancer Society.

Manuscript received 22 July 1988, in revised form 7 November 1988, and in re-revised form 23 January 1989.

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