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INFLUENCE OF VITAMIN E AND SELENIUM ON GLUTATHIONE-DEPENDENT PROTECTION AGAINST MICROSOMAL LIPID PEROXIDATION

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Abstract—A GSH-dependent microsomal protein which inhibits lipid peroxidation has been described [R. F. Burk, *Biochim. biophys. Acta* **757**, 21 (1983)]. Studies of its mechanism indicate that it scavenges free radicals. Vitamin E (α-tocopherol) and selenium are micronutrients which protect against lipid peroxidation. The effect of nutritional deficiencies of these substances on the GSH-dependent protection against rat liver microsomal lipid peroxidation was studied to determine whether GSH, selenium and α-tocopherol function through separate or shared mechanisms. In the ascorbate–iron microsomal lipid peroxidation system, there is a 1–3 min lag phase before lipid peroxidation begins. The length of the lag correlated well ($r = 0.87$) with the microsomal α-tocopherol content as measured by high pressure liquid chromatography. Thus, the selenium-deficient microsomes, which had a shorter lag than controls, had a somewhat lower α-tocopherol content. The vitamin E-deficient microsomes, which had no detectable α-tocopherol, had the shortest lag, but a distinct lag was present. Addition of 0.1 mM GSH to control microsomes prolonged the lag by 270%. In selenium-deficient and vitamin E-deficient microsomes, which had shorter initial lags, GSH addition caused 345 and 280% increases respectively. This suggests that the function of the GSH-dependent protective mechanism is unimpaired in these deficiencies. Trypsin digestion of microsomes, which abolished the lag completely and destroyed the GSH-dependent protection, had no effect on microsomal α-tocopherol content, however. These experiments illustrate the importance of two defenses against microsomal lipid peroxidation: the GSH-dependent protein which is responsible for the existence of the lag, and α-tocopherol which affects the length of the lag. They suggest that these defenses function separately to prevent peroxidation of membrane polyunsaturated fatty acids. Selenium appears to affect microsomal α-tocopherol content but to have no other effect on the microsomal lipid peroxidation system.

The existence of a GSH-dependent microsomal protein which inhibits lipid peroxidation has been reported recently [1–3]. Studies of its mechanism indicate that it functions by scavenging free radicals [2] and thus is likely to be an important membrane defense. Vitamin E is a free radical scavenger which is present in microsomes and which could be involved in this protection. In fact, it has been suggested in the past that GSH might be involved in regenerating vitamin E. A recent study supports this hypothesis [3].

The experiments reported here were carried out to investigate the possible role of vitamin E in protection against microsomal lipid peroxidation and its relationship to the GSH-dependent mechanism. The micronutrient selenium was studied too because it also has antioxidant effects *in vivo*.

MATERIALS AND METHODS

Animals. Male Holtzman rats were fed a semi-synthetic diet [4] from weaning for at least 14 weeks. The control diet contained 0.5 mg selenium/kg as Na_2SeO_3 and 100 I.U. vitamin E/kg as *dl*-α-tocopheryl acetate. The selenium-deficient diet omitted the selenium and the vitamin E-deficient diet omitted the *dl*-α-tocopheryl acetate and contained tocopherol-stripped corn oil. The rats had free access to food and water up to the time of the experiment.

Microsomal preparation and incubation. Microsomes were isolated and washed once in 0.15 M KCl as previously described [5]. The microsomes were suspended in the incubation buffer, 50 mM Tris-HCl, pH 7.5, containing 0.14 M NaCl, at an approximate concentration of 10 mg protein/ml. Incubations were carried out in open flasks maintained at 37° in a shaking water bath. The ascorbate–iron lipid peroxidation system [1] contained 0.5 mM L-ascorbic acid, 2 mM ADP and 6 μM $FeCl_3$ prepared in the incubation buffer. Microsomal protein concentration was approximately 0.5 mg/ml in the incubation flask. The total incubation volume was 5 ml.

Trypsin digestion experiments were carried out, as described previously [2], in the presence of 0.5% pyrogallol. The pyrogallol was added to prevent lipid peroxidation initiated by the trypsin digestion.

Assays. α-Tocopherol was determined by the method of Bieri *et al.* [6, 7] as modified below. Microsomes (20 mg protein) in 1.0 ml of incubation buffer were mixed with 1.5 ml of 2% ethanolic pyrogallol solution and heated at 65° for 5 min. The mixture was flushed with N_2 for an additional 5 min while heating was continued. After addition of 0.5 ml

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of 60% aqueous KOH, the tube was stoppered and mixed every minute for 5 min. The sample was then placed in ice for 20 min. α-Tocopherol was extracted into 4.0 ml of heptane after the addition of 3.0 ml of H_2O to the sample. After the addition of heptane, the sample was vortexed for 2 min and centrifuged at 600 *g* for 15 min. An aliquot of the heptane layer (3.0 ml) was removed and evaporated under N_2. The dried extracted α-tocopherol sample was dissolved in 100 μl ethanol. An aliquot was injected into a Beckman high pressure liquid chromatographic system fitted with an Ultrasphere-ODS column (4.6 mm × 25 cm). The α-tocopherol was measured at 292 nm using a Hitachi variable wavelength detector. The elution buffer contained methanol/H_2O/iso-propanol (92:8:1.5, by vol.). A standard curve was obtained by the addition of 1–5 μg *dl*-α-tocopherol to the pre-extraction microsomal suspension. The minimum amount of α-tocopherol detectable was 0.5 μg.

Thiobarbituric acid (TBA)-reactive substances were assayed as described previously [8]. Malonaldehyde bis-(dimethyl acetal) was used to prepare a standard curve. The assay mixture contained 0.1% butylated hydroxytoluene to prevent the formation of TBA-reactive substances during the assay procedure.

Protein was determined by the method of Lowry *et al.* [9] using bovine serum albumin as a standard. Fatty acid analysis of microsomes was performed as described previously [2].

Materials. L-Ascorbic acid, ADP, GSH, thiobarbituric acid, butylated hydroxytoluene, *dl*-α-tocopherol, pyrogallol, trypsin (type III), and soybean trypsin inhibitor were purchased from the Sigma Chemical Co., St. Louis, MO. The Aldrich Chemical Co., Milwaukee, WI, supplied the malonaldehyde bis-(dimethyl acetal). High pressure liquid chromatography (HPLC) grade methanol was purchased from MCB Manufacturing Chemists, Inc., Cincinnati, OH. Distilled, deionized H_2O was filtered through a 0.45 μm filter before use on the HPLC. All other chemicals used were reagent grade or better.

RESULTS

The effects of vitamin E deficiency and of selenium deficiency on the GSH-dependent microsomal protective factor which inhibits lipid peroxidation were examined. Figure 1 shows a representative experiment in which vitamin E-deficient, selenium-deficient, and control microsomes were incubated with the ascorbate–iron lipid peroxidation system in the absence and in the presence of 0.1 mM GSH. Lipid peroxidation was determined by measuring the amount of TBA-reactive substances present. The amount of lipid peroxidation and the rate of lipid peroxidation, once it started, were similar for all types of microsomes. GSH addition extended the lag prior to lipid peroxidation in all microsomes. However, selenium-deficient microsomes and vitamin E-deficient microsomes had shorter lags than did controls. The results of several experiments, summarized in Table 1, show that the length of the lag in selenium-deficient microsomes was slightly less than that in control microsomes in the absence and in the presence of 0.1 mM GSH. Vitamin E deficiency caused an even more substantial decrease in the lag prior to lipid peroxidation, but the lag was still present. Although the length of the protective lag was shorter in selenium deficiency and in vitamin E deficiency, the percent increases in the lag caused

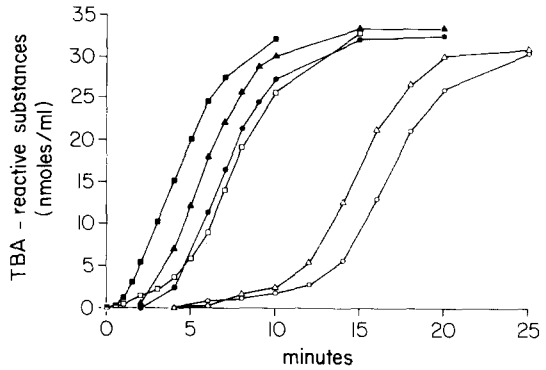

Fig. 1. Effect of GSH on ascorbate–iron microsomal lipid peroxidation. Microsomes from selenium-deficient (▲), vitamin E-deficient (■) and control (●) rat liver were incubated with 0.5 mM L-ascorbic acid, 2 mM ADP and 6 μM $FeCl_3$. The amounts of TBA-reactive substances present were measured at varying incubation times. Closed symbols represent incubations with no GSH in the flask. Open symbols represent incubations with 0.1 mM GSH in the flask. The length of the lag was determined by extrapolating a straight line from the most rapid rate of lipid peroxidation to the time axis [1].

Table 1. Effect of GSH on microsomal lipid peroxidation

Diet	N	Length of lag* (min)		% Increase
		No GSH	0.1 mM GSH	
Control	5	2.9 ± 1.0†	10.8 ± 3.2‡	272
Selenium-deficient	4	2.0 ± 0.5	8.9 ± 2.0	345
Vitamin E-deficient	4	1.0 ± 0.2†	3.8 ± 0.5‡	280

* Values are means ± S.D. All values with 0.1 mM GSH are significantly different ($P < 0.05$) from the corresponding values with no GSH added, as determined by Student's paired *t*-test.

†, ‡ Values with the same superscript are significantly different ($P < 0.05$), as determined by Student's unpaired *t*-test.

Table 2. Microsomal α-tocopherol levels*

Diet	N	α-Tocopherol (nmoles/mg protein)
Control	11	0.31 ± 0.09†
Selenium-deficient	6	0.21 ± 0.03†
Vitamin E-deficient	5	ND‡

* Values are mean ± S.D.

† Values with the same superscipt are statistically different ($P < 0.02$), as determined by Student's unpaired *t*-test.

‡ Not detectable. The minimum amount of α-tocopherol detectable was 0.06 nmoles/mg protein.

by the addition of 0.1 mM GSH were similar in both types of deficient microsomes when compared to that in control microsomes (Table 1).

The characteristics of microsomal lipid peroxidation are strongly affected by the fatty acid composition of the microsomes [10]. We determined the fatty acid composition of control, selenium-deficient, and vitamin E-deficient microsomes and found no significant differences among them (R. F. Burk and K. Patel, unpublished observations). This appears to eliminate fatty acid composition differences as a possible cause of the differences in the lag.

Because vitamin E deficiency shortened the protective lag, we measured the α-tocopherol content of the microsomes (Table 2). Selenium-deficient microsomes contained one-third less α-tocopherol than control microsomes, and α-tocopherol was undetectable in microsomes isolated from vitamin E-deficient rat liver. When results of all experiments were plotted, there was a good correlation ($r = 0.87$) between microsomal α-tocopherol content and the length of the lag before lipid peroxidation started (Fig. 2). The lowered α-tocopherol level of selenium-deficient microsomes was reflected by a slightly shorter lag before the start of lipid peroxidation. Similarly, the vitamin E-deficient microsomes with no detectable α-tocopherol had an even

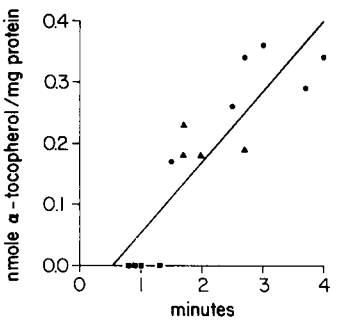

Fig. 2. Correlation between microsomal α-tocopherol and the lag prior to lipid peroxidation. Microsomes from selenium-deficient (▲), vitamin E-deficient (■) and control (●) rat liver were incubated with the ascorbate–iron lipid peroxidation system with no GSH added, and the lag prior to the onset of lipid peroxidation was determined. The α-tocopherol content of each microsomal suspension was determined by HPLC.

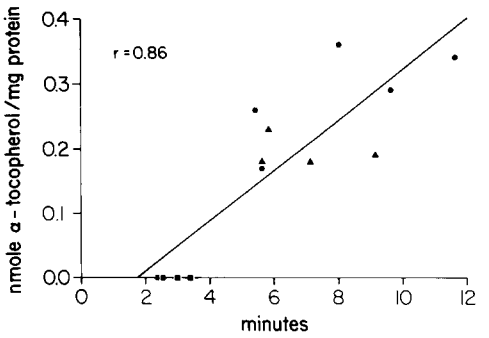

Fig. 3. Correlation between microsomal α-tocopherol and the length of time the protective lag was extended by 0.1 mM GSH. Microsomes from selenium-deficient (▲), vitamin E-deficient (■) and control (●) rat liver were incubated with the ascorbate–iron lipid peroxidation system in the presence of 0.1 mM GSH and in the absence of GSH. The difference between the length of the lag prior to the onset of lipid peroxidation in the presence and in the absence of 0.1 mM GSH is the length of time the protective lag was extended by 0.1 mM GSH. The α-tocopherol content of each microsomal suspension was determined by HPLC.

shorter, but measurable, lag. The α-tocopherol content of the microsomes also correlated with the length of time the protective lag was extended by 0.1 mM GSH (Fig. 3). Thus, the higher the α-tocopherol concentration in the microsomes, the longer was the initial lag and the longer was the extension of the lag by GSH.

While there was a significant correlation of the length of the lag with microsomal α-tocopherol content, not all the lag can be accounted for by α-tocopherol. In Figs. 2 and 3 the regression lines intercept the time axis above zero. This suggests that a portion of the lag was due to some factor other than α-tocopherol. The involvement of GSH with this other factor is suggested by the fact that the addition of GSH caused the same percent increase in the lag regardless of the microsomal vitamin E status (Table 1).

It was demonstrated previously that trypsin digestion of microsomes completely destroys the lag whether GSH is present or not [2]. In fact, lipid peroxidation begins spontaneously during trypsin digestion without the addition of ascorbate and iron and is associated with a decrease in microsomal α-tocopherol ([2], unpublished observations). To determine whether the loss of the lag period caused by trypsin digestion was associated with a loss of α-tocopherol, the trypsin digestion was carried out in the presence of the antioxidant pyrogallol to prevent the initiation of lipid peroxidation which would destroy α-tocopherol. The pyrogallol did not inhibit the action of trypsin as judged by the effect of trypsin on microsomal cytochrome P-450 content (not shown). Trypsin digestion in the presence of pyrogallol had no significant effect on microsomal α-tocopherol content. In four trypsin digestion experiments, there was no change in the α-tocopherol content of the microsomes.

This result strongly suggests that the existence of the lag was not due to the mere presence of α-tocopherol since trypsin abolished the lag without affecting microsomal α-tocopherol content. There is no question, however, that the length of the lag was affected by microsomal α-tocopherol content (Figs. 2 and 3). The present results do not rule out the possibility that GSH may be directly involved in α-tocopherol metabolism, as suggested by other workers [3]. However, they are more compatible with the presence of two protective mechanisms: the GSH-dependent protein system and α-tocopherol, which complement each other and are mutually protective. No direct effect of selenium could be detected in the microsomal lipid peroxidation system; its effect appears to be mediated through its influence on α-tocopherol levels.

DISCUSSION

It has long been recognized that rat liver microsomal membranes contain factors which inhibit lipid peroxidation. This protection has in the past been ascribed to antioxidant substances such as α-tocopherol and to nonspecific effects of membrane proteins. With the exception of a possible peroxidase role for cytochrome P-450, the enzymes which inhibit lipid peroxidation were thought to reside in the soluble fraction of the cell [11]. Thus, the recent description [1, 2] of a GSH-dependent protein which is firmly attached to the microsomal membrane and which inhibits lipid peroxidation by free-radical scavenging is of great potential importance. The microsomal membrane is particularly subject to free radical attack because of the high degree of unsaturation of its fatty acids and of the presence of the cytochrome P-450 system in it.

The present results confirm the importance of the GSH-dependent protein and of α-tocopherol in protecting against microsomal lipid peroxidation. They also show that selenium has little or no direct effect, suggesting that its antioxidant effect is mediated by a factor in some other cell fraction.

The possibility that the GSH-dependent microsomal protein is linked to α-tocopherol function is an attractive one. Vitamin E is the only vitamin for which no enzymatic role is known. The regeneration of *d*-α-tocopherol by an enzyme using the reducing equivalents of GSH would go a long way toward explaining the extraordinary *in vivo* antioxidant effect of that form of vitamin E.

Reddy *et al.* [3] have suggested that GSH regenerates oxidized vitamin E via a microsomal enzyme. They base this conclusion on the finding that GSH addition to a microsomal lipid peroxidation system has no effect when vitamin E-deficient microsomes are used. They measured lipid peroxidation at 10 min, however. Examination of Fig. 1 shows that, indeed, little effect of GSH is seen at 10 min when vitamin E-deficient microsomes are used, but that a considerable effect is noted at earlier times. Thus, while vitamin E did affect the lag, its deficiency did not abolish it.

In addition, no evidence was found in earlier work for the oxidation of GSH as a consequence of the protection, although very small amounts could have escaped detection [2]. This would appear to diminish the possibility that GSH is involved in the regeneration of α-tocopherol. If α-tocopherol interacts with an enzyme in the microsomes, specific binding sites for this substance may be found there. Adrenal gland cell plasma membranes and liver cytosol both contain high affinity α-tocopherol binding sites [12, 13]. Murphy and Mavis [14] have been unable to demonstrate such sites in rat liver, lung, heart, and brain microsomes, however.

The results presented here are interpreted to favor the hypothesis that the GSH-dependent radical-scavenging protein and α-tocopherol function independently to protect the microsomal membrane from free-radical attack. They appear to protect one another. It is conceivable that the GSH-dependent protein protrudes from the membrane and scavenges radicals at some distance from the lipid–water interface, while the α-tocopherol acts at the interface. This would make them complementary.

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