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Short communication

No reduction of α-tocopherol quinone by glutathione in rat liver microsomes

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Abstract

The cell membrane is protected against lipid peroxidation by endogenous antioxidants such as vitamin E (α-tocopherol). The oxidised form of α-tocopherol (α-tocopherol quinone) does not have this antioxidant function. However, the literature indicates that α-tocopherol quinone can be reduced to α-tocopherol *in vivo* and thereby will add to the total antioxidant potential (Moore AN, Ingold KU. Free Radic Biol Med 1997;22:931–4). We found that GSH (reduced glutathione) did not mediate the reduction of α-tocopherol quinone, either directly in solution or in rat liver microsomes fortified with α-tocopherol quinone. This renders GSH a less likely candidate for α-tocopherol quinone reduction *in vivo*. In addition, α-tocopherol quinone did not enhance GSH-dependent protection against lipid peroxidation, either in control microsomes, or in vitamin E-extracted microsomes. Indeed, α-tocopherol quinone blocked GSH-dependent protection against lipid peroxidation in vitamin E-extracted microsomes. This indicates that α-tocopherol quinone can act as a pro-oxidant.

Keywords: Lipid peroxidation; Vitamin E; α-Tocopherol; α-Tocopherol quinone; Glutathione; Pro-oxidant

1. Introduction

Reactive oxygen species (ROS) play an important role in diseases such as cancer and cardiovascular and pulmonary disorders. They are formed continuously, e.g. by the cytochrome P450 system located in the microsomes (endoplasmic reticulum) in hepatocytes. Like all biomembranes, microsomes are susceptible to damage by ROS. In particular, polyunsaturated fatty acids in the membrane are vulnerable to lipid peroxidation. These polyunsaturated fatty acids are protected against peroxidation by antioxidants such as α-tocopherol and reduced glutathione (GSH). Lipid-soluble α-tocopherol functions as a chain-breaking antioxidant. Radicals that are formed during lipid peroxidation may abstract a hydrogen atom from the OH moiety of α-tocopherol. In this way, radicals in the lipid bilayer are made inactive and α-tocopherol radicals are formed. The latter can be further oxidised to α-tocopherol quinone, which does not function as an antioxidant because it lacks the OH moiety [1,2]. Moore and Ingold have shown [3] that α-tocopherol quinone is converted to α-tocopherol *in vivo*. This implies that α-tocopherol quinone may indirectly contribute to the antioxidant status *in vivo*.

GSH protects against lipid peroxidation in cooperation with vitamin E [1,2,4–7]. It has been reported that α-tocopherol is regenerated from the α-tocopherol radical by a microsomal GSH-dependent free radical reductase (inset in Fig. 1) [1,2,4,8]. In the present study, the recycling of α-tocopherol from α-tocopherol quinone by GSH was examined both after direct interaction and under conditions where α-tocopherol quinone was present in the liver microsomal membrane. Liver microsomes were chosen since: (a) they contain an important radical-producing system; (b) the α-tocopherol content is high; and (c) once the α-tocopherol has been oxidised, the lipophilic α-tocopherol quinone is likely to be retained within the microsomes. Moreover, the microsomes contain a free radical reductase that converts the α-tocopherol radical to α-tocopherol at the expense of GSH [1,2,4,8]. Possibly this or another microsomal enzyme might be involved in a GSH-dependent reduction of α-tocopherol quinone to α-tocopherol. The effect of α-tocopherol quinone on the protection by GSH against lipid peroxidation was also studied.

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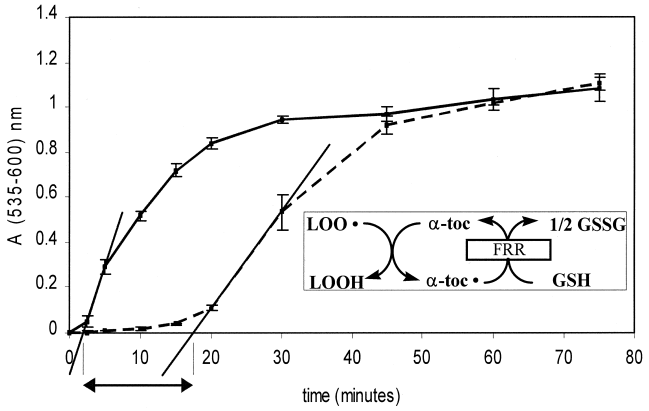

Fig. 1. Influence of GSH (1 mM) on the time-course of lipid peroxidation in control microsomes with (· · ·) and without (—) addition of GSH. Lipid peroxidation was induced by ascorbic acid (0.2 mM) and $FeSO_4$ (10 μM). Lag times, as given in Table 1, were determined as shown in the figure. Results are shown as the means (±SEM) of three experiments. Inset: Interaction of α-tocopherol (α-toc) and GSH in the protection against lipid peroxidation. The α-tocopherol radicals (α-toc•) are regenerated to α-tocopherol by GSH. This reaction is catalysed by a free radical reductase (FRR).

2. Materials and methods

2.1. Chemicals

GSH and α-tocopherol quinone were obtained from ICN Biomedicals, and D-α-tocopherol was obtained from Sigma. All other chemicals used were of analytical grade purity.

2.2. Chemical reduction of α-tocopherol quinone

α-Tocopherol quinone (0.1 mM) was incubated with GSH (0.1, 1, and 10 mM) at 25° in potassium phosphate buffer (10 mM, pH 7.4, total volume 0.6 mL). The change in the absorption spectra over time was monitored to study the reduction of α-tocopherol quinone to α-tocopherol.

2.3. Microsomes

Microsomes were prepared from male Lewis rats (CPV, Maastricht University, Maastricht, The Netherlands), 200–250 g. After decapitation, the livers were removed and homogenised (1:2, w/v) in ice-cold potassium phosphate buffer (50 mM, pH 7.4) containing 0.1 mM EDTA. The homogenate was centrifuged at 10,000 × g (20 min at 4°). Subsequently, the supernatant was centrifuged at 10,000 × g (20 min at 4°) and again at 65,000 × g (60 min at 4°). The microsomal pellet was resuspended in the phosphate buffer (2 g liver/mL) and stored at −80°. Before use, the control microsomes were thawed and washed twice (centrifugation at 115,000 × g, 40 min) with ice-cold Tris–HCl buffer (50 mM, pH 7.4) containing 150 mM KCl.

α-Tocopherol was extracted from the microsomes according to Maiorino *et al*. [9]. In short, the stored microsomes were thawed and mixed with 2 volumes of ice-cold acetone and shaken vigorously. After 5 min, the microsomes were washed twice with centrifugation at 115,000 × g (40 min at 4°). Care was taken to remove all the acetone, since it is known that acetone interferes with the method used to detect lipid peroxidation (i.e. acetone reacts with thiobarbituric acid). The pellet obtained after centrifugation of the microsomes was resuspended in ice-cold Tris–HCl buffer. α-Tocopherol quinone was added to the microsomes after the extraction with acetone. The microsomes were mixed vigorously for 1 min and washed twice (centrifugation at 115,000 × g, 40 min at 4°) with ice-cold Tris–HCl buffer.

2.4. Reduction of α-tocopherol quinone in microsomes

α-Tocopherol quinone (0.55 μM final concentration) and an excess of GSH (1 mM final concentration) was added to microsomes (2 g liver/mL) and shaken vigorously. The microsomes were incubated by shaking (190 rpm, 37°), air being freely admitted, and the α-tocopherol quinone and α-tocopherol were determined using HPLC.

2.5. Lipid peroxidation assay

Microsomes (final concentration approximately 1 mg protein/mL) were incubated at 37° by shaking (140 rpm), air being freely admitted, in Tris–HCl/KCl (50 mM/150 mM, pH 7.4). Ascorbic acid (0.2 mM) and GSH (1 mM) were neutralised with KOH before addition. Reactions were started by adding a freshly prepared $FeSO_4$ solution (10 μM). Lipid peroxidation was assayed by measuring thiobarbituric acid (TBA)-reactive material [1]. Mixing with ice-cold TBA–trichloroacetic acid (TCA)–HCl–butylhydroxytoluene (BHT) solution (2 mL) stopped the reaction in an aliquot of the incubation mixture (0.3 mL). After heating (15 min, 80°) and centrifugation (5 min), the absorbance at 535 versus 600 nm was determined. The TBA–TCA–HCl solution was prepared by dissolving 1.68 g TCA and 41.6 mg TBA in 10 mL 0.125 M HCl. BHT was dissolved in ethanol (1.5 mg/mL), and 1 mL of this solution was added to 10 mL TBA–TCA–HCl. The added chemicals did not interfere with the assay at the concentrations used.

2.6. Determination of α-tocopherol, α-tocopherol quinone, and protein

α-Tocopherol and α-tocopherol quinone were extracted from the membrane to determine the concentration in the microsomes. The extraction was performed by adding 1 mL of microsomes to 400 μL water, 2 mL ethanol, 10 μL internal standard (α-tocopherol nicotinate, 200 μg/mL). After shaking for 5 min, 3 mL hexane was added and the mixture was shaken again (10 min). The hexane layer was then evaporated and the residue was dissolved in 30 μL isopropylalcohol. The different products were quantified

using HPLC (Nucleosil 100-5 C18 column, Hewlett-Packard, eluted with 99% methanol; flow rate 2.0 mL/min) with diode array detection. α-Tocopherol and the α-tocopherol nicotinate were quantified using the absorption at 295 nm, α-tocopherol quinone using the absorption at 270 nm. Microsomal protein was assayed according to Smith *et al*. [10], using BSA as standard.

2.7. Measurement of GSH

The stability of reduced GSH during the incubation was verified according to the method developed by Ellman [11] by adding 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB, final concentration 0.6 mM) to 0.1 mM GSH solution incubated in potassium phosphate buffer (10 mM, pH 7.4) for up to 30 min. Incubations were performed with and without the addition of 0.1 mM α-tocopherol quinone. The reaction of GSH with DTNB was allowed to proceed for 5 min, after which the absorbance at 412 nm was determined. It should be noted that this assay only measures GSH and not GSSG, since glutathione reductase is not present.

3. Results

The possible reduction of α-tocopherol quinone to α-tocopherol by GSH was examined by adding different concentrations of GSH (0.1, 1, and 10 mM) to an α-tocopherol quinone solution (0.1 mM) in buffer. The change in absorption spectra was examined to monitor the reaction. The α-tocopherol quinone spectrum did not change within 30 min after addition of GSH, regardless of the GSH concentration, indicating that no reaction had occurred. Fig. 2 shows an example of such a spectrum (the interaction between 0.1 mM GSH and 0.1 mM α-tocopherol quinone is depicted). In addition, no GSH consumption, measured by the non-enzymatic GSH assay, was observed during the 30-min incubation period, confirming that no reaction had indeed taken place. The strong reductant borohydride caused the reduction of α-tocopherol quinone to α-tocopherol hydroquinone, as is also observed in the change in the absorption spectrum. For comparison, the spectrum of α-tocopherol was also depicted.

To further examine the interaction of oxidised α-tocopherol with GSH, rat liver microsomes were loaded with α-tocopherol quinone (0.55 μM) and the GSH (1 mM)-induced formation of α-tocopherol was monitored by HPLC (approximately 2000 times more GSH than α-tocopherol quinone was present in the microsomes). The membrane concentrations of α-tocopherol and α-tocopherol quinone were not altered by addition of GSH to microsomes fortified with α-tocopherol quinone. Next, the endogenous vitamin E was removed from the microsomes by acetone extraction. After extraction, the α-tocopherol quinone concentration dropped from 0.23 nmol α-tocopherol/mg protein to below the lower limit of detection (i.e. 0.036 nmol α-tocopherol/mg protein). Lipid peroxidation and the effect of GSH on this process were examined in both these extracted microsomes (with or without the addition of α-tocopherol quinone) and in control unextracted microsomes (with or without the addition of α-tocopherol quinone). Reduction of α-tocopherol quinone by GSH is expected to increase the protective effect of GSH against lipid peroxidation. We studied whether such increased protection really occurred.

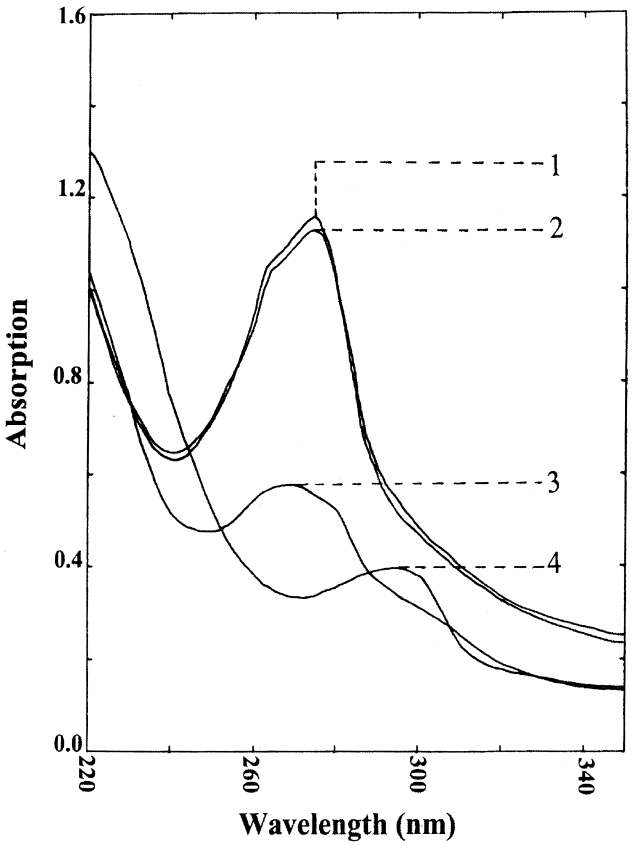

Fig. 2. The UV absorption spectra of an α-tocopherol quinone solution (0.1 mM) (blank: spectrum 1) with the addition of GSH (0.1 mM) (spectrum 2) or $NaBH_4$ (spectrum 3). Spectrum 2 was examined 30 min after the addition of GSH and spectrum 3 1 min after the addition of $NaBH_4$. Spectrum 3 was depicted to show that a reduction in α-tocopherol quinone is indeed possible. Spectrum 4 shows the absorption spectrum of α-tocopherol.

The results are listed in Table 1. In Fig. 1, the time-course of lipid peroxidation in control microsomes is depicted to explain how the lag times in Table 1 were obtained. Protection by 1 mM GSH against lipid peroxidation was clearly visible and resulted in a 14.3-min GSH-induced lag time in the control microsomes. In the extracted microsomes, protection against lipid peroxidation by GSH was reduced to 6.5 min compared to that in control microsomes. α-Tocopherol quinone (0.55 μM) addition alone had no effect on iron/ascorbate-induced lipid peroxidation. This again demonstrates that α-tocopherol quinone has no antioxidant activity on its own. α-Tocopherol quinone also had no effect on the GSH-dependent protection in control mi-

Table 1
Lag time of microsomal lipid peroxidation induced by 1 mM GSH

	GSH-induced lag time (min)	
	Without α-tocopherol quinone addition	With α-tocopherol quinone (0.55 μM) addition
Control microsomes	14.3 ± 0.4 (N = 7)	15.0 ± 0.2 (N = 5)
α-Tocopherol extracted microsomes	6.5 ± 1.0 (N = 6)	0 ± 0 (N = 4)

Lipid peroxidation was induced by 10 μM Fe^{2+} and 0.2 mM ascorbate. The lag time induced by 1 mM GSH was determined. Data are expressed as means ± SEM of at least four experiments.

crosomes, whereas it completely blocked the GSH-dependent protection in the α-tocopherol-extracted microsomes.

4. Discussion

α-Tocopherol protects against lipid peroxidation. This protection is achieved via the scavenging of lipid peroxyl radicals by α-tocopherol. The α-tocopherol radicals thus formed can be regenerated in the membrane by cytosolic antioxidants such as vitamin C or GSH (inset in Fig. 1). Nevertheless, some of the α-tocopherol radicals will not be captured by this recycling process and will be oxidised further to α-tocopherol quinone, which has no antioxidant properties [1,2].

Moore and Ingold [3] have shown that α-tocopherol quinone is reduced to α-tocopherol in man. They concluded that α-tocopherol quinone might function as an antioxidant precursor. GSH is one of the likely candidates to serve as the reductant for α-tocopherol quinone [5]. However, we found no reduction in α-tocopherol quinone by GSH either directly in solution or in fortified microsomes (Fig. 2). The presence of the cytochrome P-450 system in liver microsomes, which may serve as a potent source of free radicals, makes the α-tocopherol present in these microsomes prone to oxidation. There are several indications that the α-tocopherol radical, which is formed first, can be regenerated to α-tocopherol by a free radical reductase that uses GSH for its reducing equivalents [1,2,4,8]. As shown in this study, once the α-tocopherol had been oxidised to α-tocopherol quinone, GSH was not capable of recycling this oxidised form of α-tocopherol (Fig. 2). Apparently, the conversion of α-tocopherol quinone to α-tocopherol observed by Moore *et al*. [3] was not due to direct interaction or to a GSH-dependent reaction with α-tocopherol quinone in liver microsomes. The results of the lipid peroxidation experiments confirm that there is no significant conversion of α-tocopherol quinone to α-tocopherol by GSH in liver microsomes. If GSH had converted α-tocopherol quinone to α-tocopherol, the addition of α-tocopherol quinone to α-tocopherol-extracted microsomes would have increased the GSH-dependent lag time.

GSH-dependent protection against lipid peroxidation was clearly observed in control microsomes (Table 1). A reduction in the α-tocopherol level by extraction with acetone to a concentration below the limit of detection greatly reduced GSH-dependent protection against lipid peroxidation [12]. This was expected because GSH-dependent protection functions in part through reduction of the α-tocopherol radical, and lowering of α-tocopherol levels decreases the efficiency of the protection against radicals involved in lipid peroxidation [1,4,6,13]. α-Tocopherol quinone did not affect GSH-dependent protection in microsomes that contained α-tocopherol (control microsomes), but it did completely block GSH-dependent protection in α-tocopherol-depleted microsomes. This implies that α-tocopherol quinone can be toxic to lipid membranes of rat liver microsomes when the α-tocopherol concentration is low. A possible explanation for this effect is that α-tocopherol quinone competes with α-tocopherol for binding to the microsomal labile free radical reductase, which catalyses the reaction as shown in the inset in Fig. 1. It has been demonstrated that this microsomal free radical reductase is not specific for α-tocopherol and also interacts with α-tocopherol-like components [14,15]. Thus, α-tocopherol quinone might block the interaction of α-tocopherol with this enzyme. In this way, α-tocopherol quinone would function as an α-tocopherol antagonist. However, such an antagonistic effect of α-tocopherol quinone is only discerned when α-tocopherol levels are very low.

It has been shown previously that GSH-dependent protection is drastically reduced in partly oxidised membranes [1,2,12]. One possible explanation for this reduction is the inactivation of the free radical reductase by intermediates formed during lipid peroxidation [2]. Vitamin E consumption and, as shown in this study, the formation of α-tocopherol quinone may also contribute to the reduction of GSH-dependent protection once the process of lipid peroxidation has started. The antagonistic effect of oxidised α-tocopherol on GSH-dependent protection resembles that of oxidised glutathione, since this oxidised antioxidant also reduces GSH-dependent protection [5].

In conclusion, GSH is not able to regenerate α-tocopherol in rat liver microsomes once it has been oxidised to α-tocopherol quinone. This implies that there is no positive effect of α-tocopherol quinone on GSH-dependent protection against microsomal lipid peroxidation. In contrast, when the α-tocopherol concentration is low, α-tocopherol quinone may even reduce GSH-dependent protection, and thus act as a pro-oxidant.

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