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COMPARATIVE EVALUATION OF THE ANTIOXIDANT ACTIVITY OF α-TOCOPHEROL, α-TOCOPHEROL POLYETHYLENE GLYCOL 1000 SUCCINATE AND α-TOCOPHEROL SUCCINATE IN ISOLATED HEPATOCYTES AND LIVER MICROSOMAL SUSPENSIONS

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Abstract—The antioxidant activity of α-tocopherol polyethylene glycol 1000 succinate (TPGS) and of α-tocopherol succinate (TS) has been examined in isolated hepatocytes and microsomal fractions from rat liver. Both TPGS and TS require esterase activity to yield free α-tocopherol and, hence, antioxidant activity. TPGS and TS consistently exerted a more effective antioxidant protection than an equivalent amount of directly-added free α-tocopherol. The low antioxidant efficiency of directly added free α-tocopherol in such water-based experimental systems as used here seems to be due to its extreme hydrophobicity. TPGS, on the other hand, is an extremely hydrophilic compound that is being examined as a useful source of α-tocopherol in certain clinical situations and is here shown to be a convenient and effective source for experimental studies into lipid peroxidation and antioxidant mechanisms.

The role of free radical mediated reactions such as lipid peroxidation in a wide range of tissue injuries is a subject of great current interest. One of the main experimental approaches to investigate the role of such mechanisms in a given model of tissue injury is the use of antioxidant free radical scavenging compounds as putative protective agents. Since α-tocopherol is the major lipid-soluble chain-breaking antioxidant in biological systems [1, 2] it is the protective agent of choice in model systems where lipid peroxidation of cell membranes is under investigation. Modulation of the membrane levels of α-tocopherol is not straightforward, however. Depletion of cell membrane α-tocopherol can be achieved by feeding a deficient diet over a period of up to 15 weeks. Enrichment of liver cell membranes with α-tocopherol can be achieved by i.p. injection of a large dose of the compound before killing the animal [2, 3] but this technique is quantitatively unpredictable however, and is altogether rather unwieldy. The direct addition of α-tocopherol to isolated cells or cell membrane suspensions *in vitro* would be a far more convenient way of investigating the antioxidant effects of this compound in many experimental model systems. Unfortunately, the extreme hydrophobicity of α-tocopherol makes the reproducible direct addition of it to water-based systems such as suspensions of cells or cell membranes very difficult. Using this method, α-tocopherol added to these systems is generally much less efficient as an antioxidant than endogenous α-tocopherol. The poor antioxidant effect of α-tocopherol added to aqueous suspensions of cells and cell membranes seems to be due to an uneven dispersion that prevents its efficient dissolution in the cell membranes.

Esters of α-tocopherol that are more hydrophilic are available: α-tocopherol succinate (TS) is often used in tissue culture media and for direct addition to primary incubations of cells. Also available is α-tocopheryl polyethylene glycol 1000 succinate (TPGS) that has been the subject of little experimental attention but which has recently been shown to be very useful as a source of vitamin E in children with cholestasis who are unable to absorb normal dietary vitamin E because of the absence of bile salts [4–6]. TPGS, and possibly TS, may also be convenient sources of α-tocopherol in experimental studies *in vitro*. TS is still a very hydrophobic compound but TPGS is extremely water-soluble; both compounds require de-esterification to reveal the free phenol group that is necessary for antioxidant activity. In the present paper we have investigated the antioxidant activity of TS and TPGS in direct comparison with α-tocopherol in isolated hepatocytes and in suspensions of liver microsomes.

MATERIALS AND METHODS

Male Wistar rats, 200–250 g body wt, were obtained from Nossan (Correzzana, Italy). They were fed on semi-synthetic diet containing 40 I.U. of α-tocopherol and 12,000 I.U. of vitamin A/kg, with free access to water. Collagenase type I, α-tocopheryl succinate (TS) and bis-(*p*-nitrophenyl)phosphate (BNPP) were obtained from

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the Sigma Chemical Co. (St. Louis, MO). Alpha-tocopheryl polyethylene glycol 1000 succinate (TPGS) was a gift of Eastman Kodak Co. (Rochester, NY). Hepatocytes were isolated by the collagenase perfusion technique described in [7, 8] with minor modifications in order to preserve the glutathione content of the cells [9]. Liver cell suspensions (5×10^6 cells/mL) were incubated at 37° in stoppered 50 mL flasks in a final volume of 1–2.5 mL in a buffered medium [7] at pH 7.4. The α-tocopherol content of hepatocytes was evaluated after 30, 60, 120 and 240 min incubations in the presence of either free α-tocopherol, TPGS or TS (each at 100 μM). Alpha-tocopherol and TS were added to the cell suspension dissolved in dimethyl sulphoxide (final concentration 14 mM), while TPGS was diluted in water. Lipid peroxidation was measured as MDA production using the TBA test [8] after 1 hr incubation with ADP/iron (2.5 mM/100 μM) or cumene hydroperoxide (200 μM), or 2 hr incubation with CCl_4 (172 μM) in presence of free α-tocopherol, TPGS or TS (each at 100 μM) with or without preincubation. The effect of bis-*p*-nitro-phenyl-phosphate (BNPP), an inhibitor of non-specific esterases was studied by adding to the hepatocytes 25 μM of the compound 10 min before preincubation and incubation procedures.

Liver microsomes were prepared as described previously [10] and washed once in 0.15 M KCl. Microsomal ascorbate/iron dependent lipid peroxidation was evaluated as MDA production using the TBA test [8]: microsomes (1.5 mg protein/mL) were preincubated for 30 min with 100 μM free α-tocopherol or α-tocopherol esters followed by 10 min incubation with ascorbate–iron (500 μM/5 μM) in a system containing 37 mM KCl and 49 mM Tris buffer at pH 7.4.

Hepatocyte α-tocopherol was extracted and measured essentially as described in [1]. Aliquots of hepatocyte suspension were washed once with the incubation medium and then mixed with 2 mL of 100 mM SDS, 4 mL of absolute ethanol and 1 mL of *n*-heptane. After vortex-mixing and a brief centrifugation, the *n*-heptane layer was taken for HPLC analysis [1]. TPGS and TS did not interfere with this assay. Microsomal proteins were determined by the method of Lowry *et al.* [11].

RESULTS AND DISCUSSION

The use of α-tocopherol in water-based experimental models is fraught with problems and it is often observed that it has very low antioxidant activity when added exogenously in comparison with α-tocopherol that is endogenously present. Examples of this are seen in the papers of Cadenas *et al.* [12] and Cheeseman *et al.* [2] where the effect of α-tocopherol added to liver microsomal suspensions stimulated to peroxidise by ascorbate-iron and by NADPH/ADP-iron, respectively, was found to be much lower than that of α-tocopherol already incorporated into the membrane. Since there is no doubt that α-tocopherol is an extremely efficient antioxidant it must be concluded that in such systems α-tocopherol is not incorporated into the membrane in a way that permits interaction with lipid peroxyl radicals. This may be a consequence of the extremely

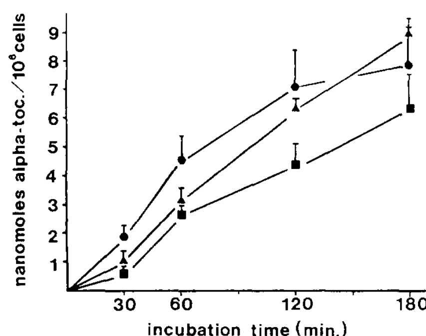

Fig. 1. Time course for the uptake of free α-tocopherol (●) TPGS (▲) and TS (■) by isolated rat hepatocytes measured as free α-tocopherol by HPLC. All additions were 100 μM. Endogenous α-tocopherol (0.799 ± 0.076 nmol/10^6 cells) was subtracted from each value. Values are means of 3–5 experiments $\pm$SE.

hydrophobic α-tocopherol molecules aggregating upon addition to water to yield micelles external to the membrane bilayer. In this paper we have investigated the possibility of using less hydrophobic derivatives of α-tocopherol as more convenient and efficient sources of α-tocopherol for experimental models *in vitro* such as suspensions of isolated cells or sub-cellular membrane fractions.

Reed *et al.* [13–15] have been foremost in investigating the role of α-tocopherol on protecting hepatocytes from oxidative stress. In those studies Reed *et al.* have mostly used TS as an exogenous α-tocopherol source and demonstrated that it can afford protection against chemical toxicity. A key point of their studies has been the critical importance of the external calcium concentration in modulating the TS effect and a recent publication [16] has emphasised this; in our study the calcium concentration was always 1 mM. In considering the value of the esters TPGS and TS as exogenous α-tocopherol sources, we have examined (a) their efficiency in supplying free α-tocopherol to target cells and cell membranes and (b) their efficiency as antioxidants (or, more properly, antioxidant precursors) in several pro-oxidant systems.

When isolated hepatocytes are incubated with free α-tocopherol, TPGS or TS, a time-dependent increase in the free α-tocopherol content of the cells as measured by HPLC was observed (Fig. 1). These data should represent, in the case of direct addition of free α-tocopherol addition, the actual uptake of α-tocopherol by the cells and, in the case of TPGS and TS addition, the release of free α-tocopherol into the cells by the action of esterases. The ability of the intact TPGS molecule to enter cells has been demonstrated by Traber *et al.* [8] using human fibroblasts, erythrocytes and an intestinal cell line. Thus TPGS may be taken up by hepatocytes as the intact molecule and hydrolysed to free α-tocopherol within the cell. The rate of α-tocopherol appearance in the cells tended to be slower using TPGS or TS compared to direct addition of free α-tocopherol (e.g. at 60 min incubation) but after 3 hr of incubation these differences were not appreciable. It is also apparent

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Table 1. MDA production stimulated in isolated hepatocytes by ADP-iron, CCl_4 or cumene hydroperoxide and the antioxidant effect of the addition of free α-tocopherol, TGPS or TS (each 100 μM) with and without pre-incubation

Treatment	nmols MDA/10^6 cells (% inhibition)		
	ADP-iron	CCl_4	Cumene hydroperoxide
Control	4.31 ± 0.31	1.68 ± 0.08	2.50 ± 0.23
Free α-tocopherol			
With pre-incubation	3.45 ± 1.04 (20%)‖	1.05 ± 0.07 (38%)‡	1.48 ± 0.40 (41%)§
Without pre-incubation	3.50 ± 0.15 (19%)‖	0.74 ± 0.31 (56%)‡	2.03 ± 0.30 (19%)‖
TPGS			
With pre-incubation	1.52 ± 0.33 (65%)*	0.41 ± 0.07 (76%)*	0.77 ± 0.29 (69%)*
Without pre-incubation	2.50 ± 0.08 (42%)§	0.47 ± 0.04 (72%)*	1.12 ± 0.25 (55%)†
TS			
With pre-incubation	1.98 ± 0.35 (54%)‡	0.70 ± 0.12 (58%)§	0.70 ± 0.18 (72%)*
Without pre-incubation	2.57 ± 0.12 (40%)§	0.39 ± 0.01 (77%)‡	1.22 ± 0.24 (51%)‡

The pre-incubation time was 1 hr, incubation with ADP-iron or cumene hydroperoxide was for a further 1 hr and with CCl_4 for a further 2 hr. Values in parentheses are % inhibition relative to controls.

Values are means ± SE of 8–12 experiments. Statistical significance of difference relative to control (Student's *t*-test): * $P < 0.001$; † $P < 0.002$; ‡ $P < 0.01$; § $P < 0.05$; ‖ not significantly different.

that, in all cases, after 3 hr of incubation the heptocytes were able to accumulate nearly 50% of the total available α-tocopherol in the incubation medium.

All three compounds afforded some degree of antioxidant effect and with all three pro-oxidant systems (Table 1). With the tocopherol esters, pre-incubation (60 min) allowed a significantly greater antioxidant effect than did simultaneous addition, except in the case of the CCl_4 system. This enhancement of effectiveness by pre-incubation was not seen with the addition of free α-tocopherol. With or without pre-incubation and with all three antioxidant forms the CCl_4 dependent system was consistently the most sensitive to inhibition, possibly because it exhibited the lowest pro-oxidant activity.

TPGS and TS consistently provided better protection against lipid peroxidation than the equivalent concentration of free α-tocopherol despite that (at least at the beginning of the incubation) less free (and therefore active) α-tocopherol would be available in the cells. In hepatocytes, depending on which pro-oxidant stimulus was used, TPGS was 1.3 to 2.9 times more effective than free α-tocopherol without pre-incubation and 1.7 to 3.3 times more effective with pre-incubation. TS was 1.2 to 2.7 times more effective than free α-tocopherol without pre-incubation. In hepatocytes TPGS tended to be more effective than TS.

In liver microsomal suspensions the better antioxidant protection afforded by the tocopherol esters relative to directly added free α-tocopherol is even more striking (Table 2). With 30 min pre-incubation TPGS inhibits ascorbate–iron dependent lipid peroxidation by 68%, TS inhibits by 47% and free α-tocopherol inhibits by a mere 3%. Microsomes clearly possess an esterase active towards the tocopherol esters since this esterase activity is essential for the antioxidant activity of the tocopherol esters to be realised by release of the free α-tocopherol. Hence, in liposomes TPGS has no antioxidant activity (data not shown). The importance of esterase

Table 2. MDA production by rat liver microsomes incubated for 10 min with ascorbate iron (500 μM/5 μM) after 30 min pre-incubation with or without α-tocopherol, TPGS or TS, each at 100 μM. Values in parenthesis are % inhibition relative to control

	nmol/min/mg protein
Control	1.15 ± 0.27
+ α-tocopherol	1.12 ± 0.31 (3%)*
+ TPGS	0.37 ± 0.11 (68%)†
+ TS	0.61 ± 0.26 (47%)*

Values are means of three experiments in duplicate ±SE.

* Not significant compared to the control (Student's *t*-test).

† Significant compared to the control (Student's *t*-test; $P < 0.05$).

activity is also evident from experiments using the esterase inhibitor BNPP in isolated hepatocytes. After BNPP treatment the hepatocytes converted much less of the tocopherol esters to free α-tocopherol and reduced their antioxidant efficacy to non-significant levels (Table 3).

Paradoxically, it is clear that TPGS and TS consistently exerted a more effective antioxidant protection than an equivalent amount of directly added free α-tocopherol. It seems likely that the apparent lack of activity of directly-added free α-tocopherol may be due in some way to its extreme hydrophobicity. We envisage that addition of α-tocopherol to a water-based system such as a suspension of cells or microsomes leads to aggregation of the α-tocopherol molecules in a micellar form with certain physico-chemical characteristics that prevent incorporation of the α-tocopherol in the cell membrane in an active configuration. From the data shown in Fig. 1 it is evident that directly added free α-tocopherol *is* taken up by the cells and at certain time-points is actually present at concentrations higher

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Table 3. α-Tocopherol levels and ADP-iron-dependent MDA production in isolated rat hepatocytes after pre-incubation with TPGS or TS: effect of bis-(*p*-nitro-phenyl) phosphate (BNPP)

	α-Tocopherol		Lipid peroxidation/(% inhibition)	
	without BNPP	with BNPP	without BNPP	with BNPP
Control	0.80 ± 0.08	0.56 ± 0.27	4.31 ± 0.31	4.98 ± 0.49
+ TPGS	2.89 ± 0.34*	0.96 ± 0.28‡	1.52 ± 0.33 (65%)*	4.07 ± 0.24 (18%)‡
+ TS	3.20 ± 0.55†	0.79 ± 0.29‡	1.98 ± 0.35 (54%)†	3.88 ± 0.23 (22%)‡

Results are nmoles of α-tocopherol or MDA per 10^6 cells and (in parentheses) percentage inhibition of lipid peroxidation by tocopherol esters; they are the mean values (±SE) from three experiments. Student's *t*-test: * $P < 0.001$; † $P < 0.01$; ‡ no significant difference compared to the control.

Hepatocytes were treated for 10 min with 25 μM BNPP, pre-incubated for 1 hr with 100 μM α-tocopherol esters and then incubated for 1 hr with ADP-iron (2.5 μM/0.1 μM) at which time lipid peroxidation was measured as MDA production.

than that of α-tocopherol derived from the esters. It must be deduced that the directly-added α-tocopherol is either not in the right location or not in an active configuration in the membrane. In the former case one may imagine that free α-tocopherol added directly to hepatocytes could accumulate in a cell membrane not undergoing lipid peroxidation while the opposite may be true for TPGS and TS. This could be due to the tocopherol esterase activity being located in the same membrane as that undergoing lipid peroxidation (e.g. endoplasmic reticulum). However, this would not explain the result seen with the microsomal suspension where α-tocopherol is added directly to the target membrane. Thus, it appears that directly added free α-tocopherol is associating with or dissolving in the membrane in an inactive configuration, possibly still in aggregates. Our data are in accord with those of Cadenas *et al.* [12] who also concluded that α-tocopherol added exogenously is ineffective because it is not properly incorporated into the membrane. Moreover, they postulated that α-tocopherol might need to be incorporated into specific sites in the membrane, i.e. near to the sites of radical production. The efficacy of TPGS and TS might be due to the *gradual* release of α-tocopherol by esterase activity at the appropriate site and rate to allow dissolution in the membrane in an active configuration, allowing rapid diffusion in the plane of the membrane and scavenging of peroxyl radicals, rather than forming inactive aggregates.

Our data are consistent with the recent report of Fariss *et al.* [16] who also found that when adding either free α-tocopherol or TS to hepatocytes the level of cell protection was greater with TS despite that a lower level of unesterified α-tocopherol was reached in the cells. They postulate that some cytoprotective effect might be due to the intact TS molecule or even the release of succinate. This to us appears to be unlikely and we believe the explanation presented here for our data is equally applicable to their observations.

Further work is required to understand precisely why α-tocopherol is ineffective when added directly to aqueous suspensions of cells or cell membranes. Meanwhile TPGS is not only finding use in the clinical situation but is also clearly a very convenient and effective source of active α-tocopherol for hepatocytes and liver microsomes *in vitro*.

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