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# INHIBITORY EFFECTS OF ISOMERS OF TOCOPHEROL ON LIPID PEROXIDATION OF MICROSOMES FROM VITAMIN E-DEFICIENT RATS

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NADPH-induced lipid peroxidation of hepatic microsomes from vitamin E-deficient rats has been used to assess the antioxidant effectiveness of dlα-. dα- and γ-tocopherol. When the tocopherols were added in ethanol to microsomes, the degree of inhibition of formation of thiobarbituric acid reactive substances (TBARS) decreased in the order dlα- > dα- > γ-tocopherol. This reflected the difference in the solubility of the tocopherols in the microsomes, dlα-tocopherol being the most soluble and γ-tocopherol the least. Using inhibition of TBARS produced per tocopherol content in microsome as a measure of antioxidant potency, the effectiveness of the isomers was γ- > dα- > dlα. Despite addition of pharmacological concentrations of the isomers, it was not possible to inhibit lipid peroxidation to the same levels as were found in microsomes from vitamin E sufficient animals. Use of ethanol as a vehicle may not allow optimum orientation of the tocopherols into the lipid bilayer.

KEY WORDS: Tocopherols, microsome, lipid peroxidation.

## INTRODUCTION

The pathogenesis of many diseases is thought to involve free radical-mediated lipid peroxidation of biological membranes.[1] Adequate dietary intake of vitamin E, a major lipid soluble inhibitor of peroxidation, is important in preventing the development of many pathogenic conditions.[2] Commercially available forms of vitamin E include dα-tocopherol and dγ-tocopherol which can be extracted from plants and dlα-tocopherol, a mixture of eight stereoisomers in approximately equal amounts which is synthesised by coupling trimethylhydrylquinone with isophytol.

In clinical conditions which may be ameliorated by vitamin E supplementation,[2] it is important to establish which form of tocopherol is the most effective antioxidant. The study reported here has assessed the antioxidant properties of d-α, dl-α and γ-tocopherol by measurement of their ability to prevent NADPH-induced lipid peroxidation of hepatic microsomes from vitamin E deficient rats.

## MATERIALS AND METHODS

Twenty-four weanling male rats of the Rowett Hooded Lister strain were randomly divided into two groups and offered, *ad libitum*, a semisynthetic diet[3] containing either 200 mg dlα-tocopherol acetate/kg (Sigma, Poole, Dorset) (+ E group) or a vitamin E content of less than 0.5 mg/kg (− E group). The selenium content of both

diets was 0.1 mg/kg added as $Na_2SeO_3$. After five weeks, the rats were anaesthetised with ether and blood was removed by cardiac puncture into heparinised evacuated tubes (Becton Dickinson, Oxford, U.K.). Harvested plasma was stored at −70°C. Following *in situ* perfusion with chilled 0.15 M KCl, the livers were removed for preparation of microsomes[4] which were adjusted to a protein content of 10 mg/ml in 0.05 M potassium phosphate buffer (pH 7.4).

Plasma and microsomal vitamin E concentration were measured by HPLC[5] using "Tocol" (BASF, Germany) as an internal standard. Test kits were used for the determination of plasma activities of pyruvate kinase (Boeringer Mannheim, Lewes, East Sussex, U.K.) and creatine kinase (Sigma, Poole, Dorset). Microsomal glutathione peroxidase activity was measured using 0.25 mM $H_2O_2$ as substrate in the presence of 5 mM reduced glutathione[6] and plasma concentrations of lipid hydroperoxides were estimated as thiobarbituric acid reactive substances (TBARS).[7]

The effects of added dlα-tocopherol (all-rac-(ordl) α-tocopherol, dα-tocopherol (RRR-α-tocopherol) and dγ-tocopherol (all-rac-γ-tocopherol) (BASF, Germany) on microsomal lipid peroxidation were assessed as follows:− 40 μl of an ethanolic solution of isomer (final concentrations 0 mM, 0.004 mM, 0.04 mM, 0.4 mM and 1.0 mM) were incubated at 37°C with stirring for 30 min in a diluted microsomal suspension (100 μl in 5.8 ml of potassium phosphate buffer, 0.05 M, pH7.4). Peroxidation was then initiated by the addition of NADPH (final concentration 0.05 mM) and 1 ml aliquots were removed at intervals for determination of TBARS.[7] Incorporation of the isomers into the microsomes was assessed by their measurement in a washed pellet prepared by centrifugation of aliquots of the incubation at 105,000 g.

Where apppropriate, comparisons were made using Students t-test.

## RESULTS

Compared with + E rats, − E rats had significantly decreased plasma and microsomal vitamin E concentrations ($P < 0.001$) and elevated plasma TBARS and pyruvate kinase and creatine kinase activities ($P < 0.001$). Microsomal glutathione peroxidase activity was similar in the two groups (Table I).

TABLE I

Plasma vitamin E concentrations, pyruvate kinase (PK) and creatine kinase (CK) activities, and thiobarbituric acid reactive substances (TBARS) and microsomal vitamin E content and glutathione peroxidase (GSHPx) activities of rats consuming diet deficient in vitamin E (− Vit. E) or supplemented with 200 mg α-tocopherol acetate/kg (+ Vit. E).

| Parameter | − Vit. E | + Vit. E |
|---|---|---|
| Plasma: | | |
| Vitamin E (μg/ml) | 0.29 ± 0.07 | 4.05 ± 0.48*** |
| PK (mU/ml) | 149 ± 3 | 46 ± 2*** |
| CK (mU/ml) | 112 ± 2 | 67 ± 2*** |
| TBARS (nmol/ml) | 8.2 ± 1.5 | 2.3 ± 0.2*** |
| Microsome: | | |
| Vitamin E (ng/mg protein) | 0.06 ± 0.02 | 0.69 ± 0.02*** |
| GSHPx (U/mg protein) | 0.05 ± 0.004 | 0.06 ± 0.01 |

Data as mean ± SE: 12 rats per group*** $P < 0.001$.

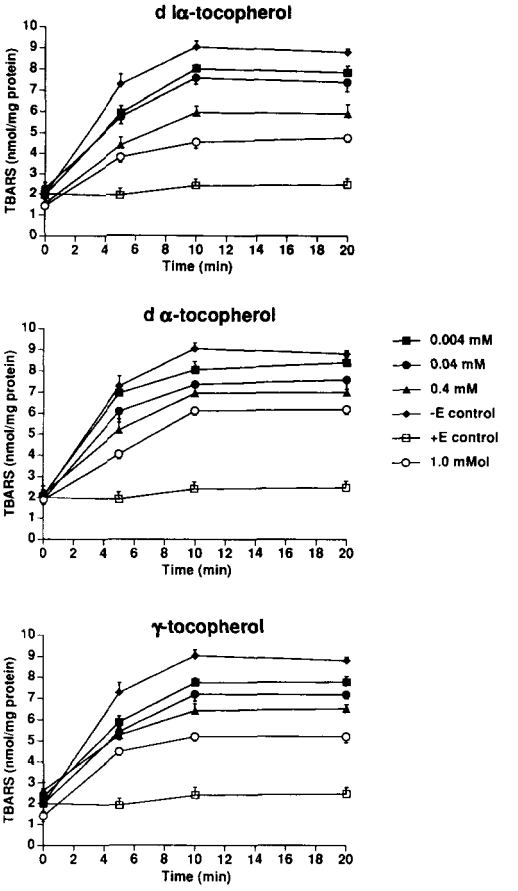


FIGURE 1 Effects of the addition of various concentrations of dlα-, dα- and γ-tocopherol on NADPH-stimulated lipid peroxidation of microsomes from vitamin E-deficient (−E) rats. Also shown are data for −E microsomes without exogenous tocopherol (−E control) and for microsomes from vitamin E-supplemented (+E control) rats.

When incubated in the presence of NADPH, hepatic microsomal fractions from −E rats produced TBARS at a greater rate than those from +E rats. This difference was significant ($P < 0.001$) within 5 min of the addition of NADPH. Preincubation of −E microsomes with increasing concentrations of dlα-, dα- or γ-tocopherol progressively decreased the rate and extent of TBARS formation (Figure 1). Compared with −E microsomes incubated without exogenous tocopherol, maximum peroxidation was reduced by 47%, 42% and 31% for dlα-, γ-, and dα-tocopherol, respectively (Figure 2). In no case was TBARS formation reduced to the level observed in microsomes from vitamin E sufficient rats (Figure 2). Addition of isomers to microsomes derived from +E rats had no effect on peroxidation (data not shown).

There were marked differences in the degree of incorporation of the isomers into the microsomes (Figure 3). For example, at a concentration of 1 mM in the external medium, the microsomal uptake of the dlα- form was 2.5 and 27 fold greater than that

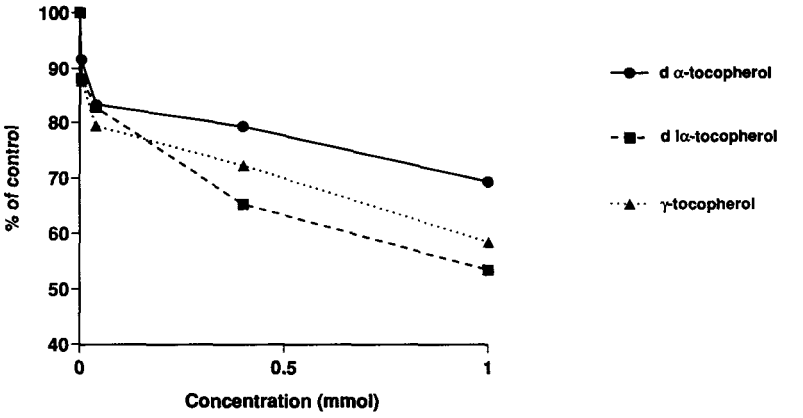


FIGURE 2 The relationship between concentration of tocopherol isomers in the suspension and its effectiveness in reducing microsomal lipid peroxidation. Results are calculated as a percentage of the control value which is the concentration of TBARS produced by −E microsomes without exogenous tocopherol after 20 min incubation.

of the dα- and γ-isomers respectively. Consequently, when the efficiency of the antioxidant isomers is compared on the basis of their concentration in the microsomes rather than in the medium, the γ-tocopherol is the most potent. Thus the microsomal concentrations of γ-, dα- and dlα- required to reduce TBARS formation from 8 to 5 nmoles/ml are 60, 740 and 1300 μg/mg protein respectively (Figure 4).

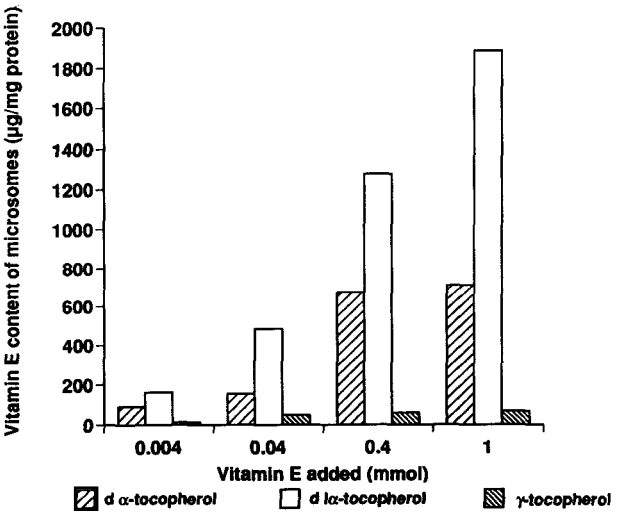


FIGURE 3 The relationship between final concentration of tocopherol isomers in the incubations medium and the actual concentration of the isomers in recovered microsomes. Measurements of microsomal tocopherols were made following 30 min incubation of microsomes from vitamin E deficient rats with exogenous tocopherols.

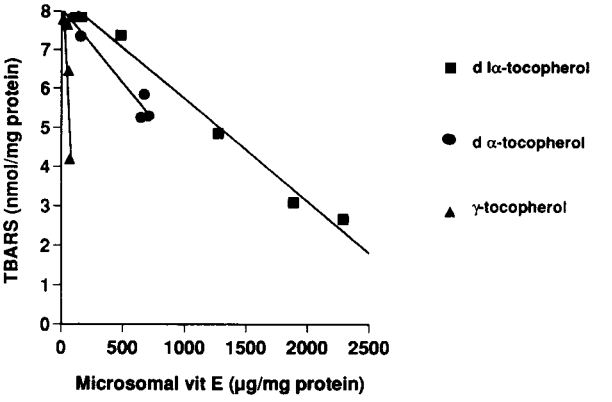


FIGURE 4 Effect of added tocopherol isomers on maximum TBARS formation by microsomes from vitamin E deficient rats. Correlation coefficients relating TBARS to microsomal vitamin E are: dlα-, $r = 0.993$, $P < 0.01$: dα-, $r = 0.976$, $P < 0.01$: γ-, $r = 0.790$, $P < 0.05$.

## DISCUSSION

Increased plasma pyruvate kinase and creatine kinase activities and TBARS concentrations indicated that the rats fed the vitamin E-deficient diet had marked free radical-mediated cell membrane damage. Susceptibility to free radicals was further emphasised by the greatly enhanced peroxidation of the microsomal preparations from the vitamin E-deficient rats. This peroxidation can be ascribed to the decreased microsomal vitamin E content; microsomal glutathione peroxidase activity was the same in the vitamin E-deficient and supplemented rats. The microsomal glutathione peroxidase activity may reflect residual cytoplasmic or a membrane-associated form of the enzyme.[8]

Of the 3 isomers, dlα-tocopherol appeared to be the most effective at inhibiting lipid peroxidation insofar as less of this isomer was required in the medium to reduce TBARS to a particular level. However, this can be attributed to the greater uptake of the isomer by the microsomes. When the potency of the isomers is based on their concentration within the microsome, γ-tocopherol is clearly much more effective than the others. This observation may be of limited biological relevance as following absorbtion it is rapidly removed from the circulation by the liver.[9]

Despite the addition of pharmacological concentrations of the tocopherols to the microsomes from − E rats, it was not possible to reduce the formation of TBARS to the levels found in microsomes from the vitamin E-sufficient animals. In such microsomes peroxidation was minimal despite the vitamin E content being only 1% of that in the microsomes to which exogenous tocopherol was added. Similar results have been obtained when microsomal lipid peroxidation is initiated by ascorbate/ADP/$Fe^{3+}$.[10] Specific plasma and cytosolic transporters and membrane receptors may be required to achieve the correct alignment of the tocopherol molecule in the cell membrane.[11,12] The use of ethanol as a vehicle may result in the partitioning of the tocopherol in the membrane so that the functional hydroxy group of the chromanol ring is not in the appropriate position to donate a hydrogen to the adjacent peroxidising

phospholipids. A failure of vitamin E isomers to orientate correctly could be further exacerbated by prior peroxidative damage to the membrane because of the vitamin E deficiency. Additionally, the vitamin E deficiency may deplete putative vitamin E receptors or binding proteins in the membrane.

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