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EFFECTS OF α-TOCOPHEROL ON CARBON TETRACHLORIDE METABOLISM IN RAT LIVER MICROSOMES

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α-tocopherol is the major lipid-soluble radical-scavenging antioxidant in rat liver. It has long been used as a putative protective agent in CCl_4 induced liver injury but with variable results. We have used α-tocopherol loaded rat liver microsomes to study the effect of this vitamin on CCl_4 metabolism *in vitro*. As expected, α-tocopherol inhibits CCl_4-dependent microsomal lipid peroxidation and, at a very high concentration, will inhibit the covalent binding of $CCl_3\cdot$ to microsomal protein by up to 50%. No inhibitory effect was observed towards $CCl_3\cdot$ production as measured by the electron spin resonance technique of spin-trapping but this apparent discrepancy may represent a limitation of the technique. The high levels required to inhibit covalent binding probably preclude the likelihood of α-tocopherol significantly affecting that phenomenon at endogenous concentrations but may be relevant to other experiments employing high doses of α-tocopherol as an experimental hepatoprotective agent.

KEY WORDS: α-tocopherol, carbon tetrachloride, lipid peroxidation, covalent binding, spin-trapping.

INTRODUCTION

Administration of carbon tetrachloride (CCl_4) to a rat produces centrilobular necrosis and fat accumulation in the liver. This hepatotoxicity of CCl_4 is dependent upon its metabolism by the cytochrome P-450 system of the endoplasmic reticulum to a reactive intermediate, the trichloromethyl free radical, $CCl_3\cdot$.[1,2] Subsequent to this activation step, peroxidative degradation of the polyunsaturated fatty acids of the endoplasmic reticulum is initiated and metabolites of CCl_4 bind covalently to membrane lipids and proteins. It is now considered that $CCl_3\cdot$ is largely responsible for the covalent binding to macromolecules and that the more electrophilic trichloromethylperoxyl free radical ($CCl_3O_2\cdot$), which is produced from $CCl_3\cdot$ extremely rapidly under aerobic conditions, is the intermediate responsible for initiating CCl_4-dependent lipid peroxidation.[2] The respective roles of covalent binding and lipid peroxidation in CCl_4-induced liver damage have long been a matter of debate and it is now generally held that both processes are of major significance.[1,3]

The effects of antioxidants on CCl_4-induced liver injury have been studied over many years in attempts to confirm the role of lipid peroxidation in CCl_4 hepatotoxicity and to discriminate between cytotoxic phenomena linked to that process and those linked to binding.[1,3] The major lipid-soluble radical-scavenging antioxidant of the hepatic endoplasmic reticulum is α-tocopherol[4] and this compound has been tested

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CCl_4 liver injury in experiments over the last 35 years.[1] The results have been equivocal and the considerable variety of methods used has made comparisons extremely difficult. The most recent data on this topic are those of Corongiu *et al.*[5] and Poli *et al.*[6] each of whom used an identical technique of α-tocopherol pre-treatment that results in a large hepatic concentration of the vitamin. Corongiu *et al.* confirmed that α-tocopherol inhibits CCl_4-induced lipid peroxidation *in vivo*.[5] Poli *et al.* showed that isolated hepatocytes from α-tocopherol pre-treated rats were protected from CCl_4-induced cell death and lipid peroxidation whilst having identical levels of covalently bound CCl_4 metabolites to control cells.[6] Moreover, α-tocopherol dosed rats were protected from CCl_4-induced liver necrosis, as measured by leakage of liver cell enzymes into the plasma, for at least 24 h. We have used the same α-tocopherol dosing regime to produce rat liver microsomes considerably enriched in α-tocopherol and show that if high concentrations of the vitamin are present then it is capable of inhibiting not only CCl_4-induced lipid peroxidation but also the covalent binding of CCl_4 metabolites to microsomes *in vitro*.

METHODS

Male Wistar rats, body wt. 200–250 g (Charles River Ltd., Margate, UK) were used throughout. Where appropriate, rats were dosed with α-tocopherol (100 mg/kg body wt., i.p.) as described by Pfeifer and McCay[7] 16 h before sacrifice and at the same time food was removed from both controls and treated animals. Liver microsomes were prepared as described previously[8] and washed once by resuspension and recentrifugation in 0.1 M Tris: HCl, pH 7.4. The washed pellets were stored for 1–3 days at −70 °C before the assays were carried out when they were resuspended in 0.15 M KCl such that one ml was equivalent to one gram wet weight of liver. CCl_4-stimulated lipid peroxidation (malonaldehyde production) and the covalent binding of $[^{14}C]$-CCl_4 to microsomal protein were determined simultaneously by incubating microsomes and $[^{14}C]$-CCl_4 (Amersham International, Amersham, UK) with a source of NADPH aerobically at 37 °C as previously described[9,10] except that an incubation time of 10 mins was used. Malonaldehyde was determined in the protein-free supernatant[9] and irreversibly-bound $[^{14}C]$-CCl_4 measured in the protein pellet after solvent washing.[10] Using an essentially identical incubation medium, the $CCl_3\cdot$ radical was trapped with 15 mM α-phenyl-N-tert-butylnitrone (PBN) and the adduct extracted into toluene (0.5 volumes) after 5 min incubation under hypoxic conditions. After a brief centrifugation (800 g × 5 mins), the toluene layer was analysed by electron spin resonance spectrometry at −70 °C using a Bruker ER200D X-band spectrometer operating with 100 kHz modulation, and a Bruker ER411VT variable temperature unit.

Ethoxycoumarin deethylase was measured as previously described[11] but omitting ethanol. NADPH/ADP-iron-stimulated lipid peroxidation was measured as oxygen consumption as previously.[12] Microsomal α-tocopherol was extracted and measured essentially as described by Burton *et al.* for liver homogenates[13] with some modification. One ml of microsomal suspension (20 mg protein) was mixed with one ml of sodium dodecyl sulphate (50 mM) followed by two mls of absolute ethanol; one ml of n-heptane was added and the sample was vortex-mixed for 1–2 mins. After a brief centrifugation, the n-heptane layer was taken for analysis by HPLC. Samples (100 μl) were applied to an S5-nitrile column (25 cm × 4.6 mm; Anachem Ltd., Luton, UK);

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TABLE I
Effect of α-tocopherol dosing on CCl_4 metabolism, lipid peroxidation and ethoxycoumarin deethylase activity in rat liver microsomes *in vitro*

Parameter	Control		Treated	
α-tocopherol (nmols/mg protein)	0.5 ± 0.1	(6)	6.5 ± 3.0*	(6)
CCl_4-stimulated MDA production (nmols/mg/10 min)	3.98 ± 1.35	(6)	0.41 ± 0.64*	(6)
Covalent binding of [^{14}C]-CCl_4 to protein (nmols/mg/10 min)	2.6 ± 0.4	(6)	1.3 ± 0.4*	(6)
$CCl_3\cdot$-spin trapping (signal height)	17.5 ± 4.8	(6)	23.3 ± 5.6**	(6)
Ethoxycoumarin deethylase (nmols/mg/min)	0.27 ± 0.06	(6)	0.23 ± 0.05**	(4)
Induction period, NADPH/ADP-Fe-stimulated lipid peroxidation (mins)	0.6 ± 0.3	(6)	> 30	(6)

Values are mean ± standard deviation with number of animals in parentheses.
Treated animals received 100 mg/kg α-tocopherol i.p. as described in Methods.
* $P < 0.001$; ** N.S. (student's t test)

the mobile phase was hexane:isopropanol (99:1, v/v) pumped at 1.8 ml/min and the U.V. detector was set at 295 nm. The concentrations of α-tocopherol were determined by reference to injections of standard solutions of α-tocopherol and by use of a LDC CI10B computing integrator. Microsomal protein was determined by the method of Lowry *et al.*[14]

RESULTS AND DISCUSSION

Pre-treatment of rats with α-tocopherol at 100 mg/kg body wt. according to the method of Pfeifer and McCay[7] results in considerable enrichment of hepatic microsomal α-tocopherol. On average, the liver microsomes of the treated rats contained 13 times more α-tocopherol than those of the control rats (Table I), but this figure conceals considerable variation in the degree of loading (5-fold to 20-fold). For this reason, an analysis of the precise concentration of microsomal α-tocopherol actually achieved is to be recommended when using this method. However, while the variability of α-tocopherol loading may be sometimes inconvenient, this method is certainly much less problematic than attempts to add α-tocopherol to microsomes (or hepatocytes, liver mitochondria etc) by various methods *in vitro*.[15]

Loading with α-tocopherol strongly depresses microsomal lipid peroxidation induced by the NADPH/ADP-iron system as measured by MDA production[7] or by oxygen consumption;[4] (Table I) with the latter method providing a convenient and rapid check for α-tocopherol incorporation. In the present study, CCl_4-stimulated lipid peroxidation was decreased by α-tocopherol dosing to about 10% of control levels. Lipid peroxidation dependent upon NADPH (in the absence of CCl_4 or ADP-iron) was inhibited to a similar degree (data not shown). In all these microsomal lipid peroxidation systems the principal action of α-tocopherol is to act as a chain-breaking antioxidant, scavenging lipid peroxyl radicals and being consumed in the process.[16] In the CCl_4 system, α-tocopherol could also scavenge the proposed initiating radical $CCl_3O_2\cdot$ with which it reacts extremely rapidly ($k = 5 \times 10^8 M^{-1} sec,^{-1}$),[17] though the greater relative concentration of fatty acids, with which $CCl_3O_2\cdot$ also reacts extremely rapidly ($k = 2 - 7 \times 10^6 M^{-1} sec^{-1}$)[18]

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may make the reaction between $CCl_3O_2\cdot$ and α-tocopherol an inefficient competitive process. As well as inhibiting CCl_4-stimulated lipid peroxidation in microsomes *in vitro*, this method of α-tocopherol dosing also inhibits CCl_4-induced lipid peroxidation in liver endoplasmic reticulum *in vivo*, as shown by second derivative spectroscopy of diene conjugates by Corongiu *et al.*[5]

Measurement of the covalent binding of [^{14}C]-CCl_4 to microsomal protein is probably the most reliable quantitative index of $CCl_3\cdot$ production.[2] In this study, α-tocopherol enrichment of the microsomes inhibited covalent binding to protein by 50%. This degree of inhibition was approximately the same in microsomes containing 4.5 nmol/mg protein α-tocopherol as in microsomes containing more than double this level and may thus represent the maximum achievable inhibition of this parameter by α-tocopherol. In studies with isolated hepatocytes loaded with α-tocopherol by the same technique used here, Poli and colleagues have found no effect on the covalent binding of [^{14}C]-CCl_4 to whole cell protein, relative to hepatocytes from control animals.[6] Whether this discrepancy is a function of the different systems (microsomes versus whole cells) or due to a lower level of α-tocopherol incorporation into the cell membranes in the hepatocyte study can only be a matter of speculation. No effect of α-tocopherol pretreatment on the covalent binding of [^{14}C]-CCl_4 to liver microsomal lipids *in vivo* was found by Benedetti *et al.*[19] Clearly, it is difficult to compare data with the present study and it may be of interest in the future to compare the effect of α-tocopherol on covalent binding of [^{14}C]-CCl_4 to lipid and to protein.

Determination of $CCl_3\cdot$ production by measurement of the concentration of PBN-CCl_3 adduct by ESR spectrometry showed poor correlation with either CCl_4-stimulated lipid peroxidation or with covalent binding, and loading of microsomes with α-tocopherol did not consistently modify the quantity of spin adduct formed. McCay and colleagues have reported that α-tocopherol pretreatment enhances CCl_3-PBN adduct formation *in vivo*, probably by protecting the enzymes responsible for $CCl_3\cdot$ production from inactivation.[20]

In the present study there was an apparent increase in spin adduct formation in the α-tocopherol-loaded microsomes but this was not statistically significant. Previous studies in this laboratory[21] demonstrated a concentration-dependent reduction in the amount of CCl_3-PBN adduct when α-tocopherol was added *in vivo* to isolated hepatocytes but this may have been due to direct reduction of the spin adduct.

It seems unlikely that α-tocopherol loading actually diminishes the rate of CCl_4 activation of $CCl_3\cdot$ since the overall activity of the P-450 system, as evidenced by ethoxycoumarin deethylase activity, is unchanged by this treatment.

The above results seem to indicate that α-tocopherol, in addition to inhibiting CCl_4-stimulated lipid peroxidation by scavenging lipid peroxyl radicals may inhibit covalent binding of [^{14}C]-CCl_4 by scavenging $CCl_3\cdot$ radicals. The rate constant for the reaction of α-tocopherol with $CCl_3\cdot$ is low ($< 10^5\,M^{-1}\,sec^{-1}$) and one might expect, as indeed seems the case, that α-tocopherol would need to be present at very high concentrations to compete with membrane proteins for this radical. Therefore physiological concentrations of α-tocopherol are unlikely to significantly modify the course of this process. The inability of α-tocopherol to decrease CCl_3-PBN adduct formation is probably due to the high concentration in the system of PBN (15 mM) which effectively competes with the vitamin for $CCl_3\cdot$.

An alternative explanation for the inhibition of covalent binding is that α-tocopherol "repairs" a CCl_3-protein radical adduct and prevents reaction with a second $CCl_3\cdot$ radical:

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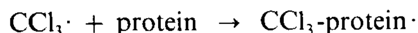
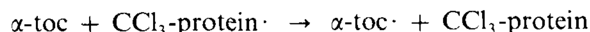


$$CCl_3\cdot + \text{protein} \rightarrow CCl_3\text{-protein}\cdot$$

$$CCl_3\cdot + CCl_3\text{-protein}\cdot \rightarrow CCl_3\text{-protein-}CCl_3 \text{ (radical/radical reaction)}$$



$$\alpha\text{-toc} + CCl_3\text{-protein}\cdot \rightarrow \alpha\text{-toc}\cdot + CCl_3\text{-protein}$$

$$\alpha\text{-toc}\cdot + CCl_3\text{-protein}\cdot \rightarrow \text{molecular products}$$

Clearly, α-tocopherol is primarily an antioxidant and only very high concentrations can significantly modify covalent binding of [^{14}C]-CCl_4 metabolites. Nevertheless, the present study has shown that very high doses of α-tocopherol, as used in some experimental models, may not necessarily discriminate between those cytotoxic effects dependent on lipid peroxidation and those dependent on covalent binding unlike the situation observed with promethazine.[1,3] The method of pre-treatment used here can inhibit CCl_4-stimulated lipid peroxidation *in vivo*[5] and protects against CCl_4-induced liver necrosis *in vivo*.[6] Since the levels of hepatic α-tocopherol vary considerably using this method it may be of benefit to repeat these experiments *in vivo* simultaneously measuring parameters of lipid peroxidation, covalent binding and cell damage and relating them to actual α-tocopherol concentrations.

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