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IN VITRO AND *IN VIVO* STUDIES OF THE EFFECT OF VITAMIN E ON MICROSOMAL CYTOCHROME P450 IN RAT LIVER

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Abstract—Administration of a diet supplemented with 0.06% vitamin E acetate to male rats over a 6-week period doubled hepatic microsomal stores of α-tocopherol over those in control (vitamin E adequate) rat liver. Total cytochrome P450 content and NADPH–cytochrome P450 reductase activity were significantly elevated in hepatic microsomes from vitamin E-supplemented rats to 111% and 123% of respective control values. Androstenedione 16α-hydroxylase activity was increased in these fractions (2.57 ± 0.31 nmol product/min/mg protein vs 1.81 ± 0.38 in controls) whereas activities of the 6β-, 7α- and 16β-hydroxylase pathways were unchanged. Immunoquantitation of the microsomal 16α-hydroxylase, P450 IIC11, indicated a corresponding increase in the hepatic content of the enzyme. In view of the established antioxidant role of tocopherols, the effects of dietary vitamin E manipulation on the concentration of protein sulphydryl groups and the susceptibility of microsomes to ferric sulphate–ADP–NADPH–mediated lipid peroxidation were also assessed. Dietary supplementation did not influence microsomal protein sulphydryl content (68 ± 10 nmol glutathione equivalents/mg protein) but decreased the extent of lipid peroxidation produced by the ferric sulphate–ADP–NADPH system *in vitro*. Further *in vitro* experiments demonstrated that vitamin E acetate (2 μM) protected protein sulphydryl groups and lipids against peroxidation in control microsomes and partially reduced the associated losses of P450-mediated steroid hydroxylase activities. Western immunoquantitation of P450 IIC11 revealed that exogenous vitamin E acetate protected completely against peroxidation-induced apoprotein loss. These studies establish that the *in vitro* protective effects of vitamin E acetate against sulphydryl and lipid peroxidation extend to protection of the P450 apoprotein but that enzyme activity is only partially protected. This finding suggests that peroxidation-dependent loss of P450 *in vitro* is mediated by haem degradation from the P450 holoenzyme and is not directly related to lipid/sulphydryl oxidation. In contrast, the *in vivo* effects of dietary vitamin E on drug metabolizing enzymes are regulatory in nature and are unrelated to effects on lipid peroxidation.

It has been proposed that proliferation of lipid peroxides may result in significant membrane damage and altered cellular function [1]. Dietary tocopherols may protect cellular constituents, including membrane lipids and protein sulphydryl groups by virtue of their potent antioxidant actions [2]. More recently it has been suggested that α-tocopherol may act in conjunction with other dietary micronutrients such as selenium to facilitate the cellular defence against toxic reactive molecules [3]. That is, there are several cellular protection systems against active oxygen species including tocopherols, the selenium-dependent glutathione peroxidase and glutathione itself [4].

It has also been demonstrated that dietary vitamin E deficiency is associated with decreased activity of the hepatic microsomal cytochrome P450-dependent mixed-function oxidase system [5, 6]. These enzymes convert a range of lipophilic agents, such as steroids and carcinogens to polar metabolites [7–10]. In the cases of the carcinogens, P450s are responsible for activation to the proximate toxic species [8–10]. Multiple forms of P450 have been isolated from mammalian liver and are regulated independently by hormonal and other factors [11, 12]. Nutritional factors exert profound influences upon microsomal P450 and drug oxidation [13] but, at present, little information is available concerning the relationship between nutrition and the regulation of specific P450 enzymes. In this study the *in vitro* and *in vivo* effects of vitamin E on hepatic microsomal P450 and related drug oxidations were assessed. *In vitro* data demonstrated that exogenous vitamin E supplementation partially protects against lipid peroxidation and associated losses of protein sulphydryl groups and P450 enzyme activity but is completely effective in preserving the P450 apoprotein. *In vivo* (dietary) supplementation with vitamin E decreased the susceptibility of microsomal fractions to lipid peroxidative stress and produced an increase in the microsomal content and activity of the principal male-specific P450 IIC11 in rat liver.

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MATERIALS AND METHODS

Animals. Weanling male Wistar rats were used in experiments dealing with dietary manipulation and were held in wire cases under uniform conditions.

Animal diets. The composition of the control diet was based on that described by Wolf *et al.* [14]: lactic casein 24.0% (H.E. Cottee Pty Ltd, Kirribilli, Australia), sucrose 53.5% (CSR Limited, Milsons

Point, Australia), cottonseed oil 15%, choline chloride 0.2%, cornstarch 0.8% (Sigma Chemical Co., St Louis, MO, U.S.A.), salt mixture 5.0% (composition: $CaCO_3$ 53.5%, $MgCO_3$ 2.5%, $MgSO_4$ 3.2%, NaCl 6.8%, KCl 11%, KH_2PO_4 20.9%, KI 0.0079%, $MnSO_4$ 0.035%, $Al_2(SO_4)_3$ 0.011%, K_2SO_4 0.056%, NaF 0.099%, $Fe_3(PO_4)_2$ 2%, $CuSO_4$ 0.089%; analytical reagent grade salts supplied by Ajax chemicals, Sydney, Australia) and vitamin mixture 1.0% [composition: retinyl acetate 25 I.U., riboflavin 0.15%, calcium pantothenoate 0.4%, nicotinic acid 1%, pyridoxine hydrochloride 0.06%, biotin 0.006%, folic acid 0.04%, 2-methyl-1,4-naphthoquinone 0.04%, vitamin B12 0.5 mg%, inositol 2%, *p*-aminobenzoic acid 0.06%, cholecalciferol 250 units, glucose (diluent) to 100%; vitamins supplied by Roche Pty Ltd, Dee Why, Australia or Sigma]. The control diet contained 0.007% of α-tocopherol, derived most probably from the cottonseed oil, whereas the vitamin E-supplemented diet contained 0.06% of α-tocopherol; standard commercial rat pellets (Young Stock Feeds, Young, Australia) contain 0.006% of the vitamin. Animals received water and either the control or vitamen E-supplemented diet *ad lib.* over a 6-week period.

Preparation of microsomal fraction. Rats were fasted overnight and killed the next morning by exsanguination under ether anaesthesia. Blood was collected by aortic puncture and the livers were removed. Livers were perfused with saline and homogenized in 10 mM potassium phosphate buffer (pH 7.4, containing 0.25 M sucrose and 1 mM EDTA). Washed hepatic microsomes were isolated by differential ultracentrifugation and the final pellets were resuspended in 50 mM potassium phosphate, pH 7.4, containing 20% glycerol and 1 mM EDTA, and stored at −70° until required.

Chemicals. [^{14}C]Androst-4-ene-3,17-dione (androstenedione) and [^{14}C]testosterone were from Amersham Australia. Reference standards were from Sigma, Steraloids (Wilton, NH, U.S.A.) or the MRC Steroid Reference Collection at Queen Mary's College (London, U.K.). Other chemicals and biochemicals were obtained from Sigma, E. Merck (Darmstadt, Germany) or Boehringer-Mannheim (Sydney, Australia). Solvents and other chemicals were obtained from Ajax Chemicals and were at least analytical reagent grade.

Enzyme assays. Microsomal protein was estimated by the Lowry method [15] with bovine serum albumin as standard. Microsomal P450 was determined by the procedure of Omura and Sato [16] and NADPH-P450 reductase activity by the method of Strobel and Dignam [17].

Microsomal steroid hydroxylation was performed essentially as documented previously [18]. The K_m (Michaelis constant) and V_{max} (maximal reaction velocity) of testosterone hydroxylation pathways in hepatic microsomal fractions from control and vitamin E-supplemented rats were determined in incubations (5 min) that contained varying concentrations of steroid substrate, protein (0.25 mg/mL) and NADPH (1 mM). In these experiments the incubations were conducted in potassium phosphate buffer (0.1 M, pH 7.4) containing EDTA (1 mM). In experiments concerned with the effects of lipid peroxidation on microsomal testosterone metabolism, the reactions were undertaken in Tris buffer (0.5 M, pH 7.4) containing no EDTA.

Products of testosterone metabolism were extracted with chloroform and applied to TLC plates (Merck silica gel; 60 F_{254}, heated for 15 min at 100° before sample application). Plates were developed in the solvent system dichloromethane:acetone, 4:1 and then in chloroform:ethyl acetate:ethanol 4:1:0.7 (for the separation of testosterone metabolites) [19] or twice in the system chloroform:ethyl acetate 1:2 (for the separation of androstenedione metabolites) [19]. Individual steroid metabolites were identified by comigration with authentic standards (using UV light) and were then scraped into scintillation vials for quantitation of radioactivity (ACS II, Amersham Australia).

P450 purification and preparation of anti-P450 antiserum. The isolation of P450 IIC11 (previously termed P450 UT-A) from untreated male rat hepatic microsomes and preparation of rabbit anti-rat anti-P450 IIC11 IgG have been described [20]. As reported previously, the IgG preparation preferentially inhibits steroid 16α-hydroxylase activity (catalysed by P450 IIC11) and recognises a single antigen in male rat liver, but does not cross-react with any antigens in female rat hepatic microsomes [20]. Purified P450 IIIA1, isolated from phenobarbital-induced rats [21], and the rabbit anti-rat P450 IIIA1 IgG were donated by Dr A. Åstrom, University of Stockholm. Anti-P450 IIIA1 IgG cross-reacts with the male-specific constitutive P450 IIIA2 and was used to immunoquantitate IIIA2 in male rat liver (P450 IIIA1 is not expressed in uninduced rat liver) [22].

Gel electrophoresis and Western immunoblotting. Sodium dodecyl sulphate electrophoresis (in 7.5% polyacrylamide gels) was performed by the method of Laemmli [23] with the exception that the concentrations of Tris and glycine in all buffers and samples were doubled to improve the resolution of P450s [24].

After electrophoresis, proteins were transferred to nitrocellulose sheets [25] which were then subjected to sequential washing, incubation with the appropriate anti-P450 IgG and incubation with ^{125}I- and horseradish peroxidase-linked donkey anti-rabbit IgG as described [20]. The nitrocellulose was dried and immunoblots corresponding to the P450 (recognized from peroxidase staining) were excised for γ quantitation. Standard curves were constructed using purified P450s.

Lipid peroxidation assay. The assay employed was similar to that described by Takayanagi *et al.* [26]. The microsomal protein concentration was 0.75 mg/mL, and other reaction components were ferric sulphate (250 μM), adenosine diphosphate (2 mM) and NADPH (0.5 mM) in Tris buffer (0.5 M, pH 7.4). Reactions were terminated after a 15 min incubation, centrifuged at 2000 *g* for 10 min and 2-thiobarbituric acid (25 mM) was added. After incubation at 80° for 20 min the formation of malondialdehyde-reactive products was monitored from the absorbance at 535 nm [27].

Lipid peroxidation and cytochrome P450 IIC11

Table 1. Dietary vitamin E manipulation: hepatic α-tocopherol content and serum hormone concentrations in male rats

Diet	Hepatic α-tocopherol (nmol/mg protein)	Serum hormone (nM)			
		Testosterone	Oestradiol	Triiodothyronine	Thyroxine
Control (vitamin E adequate)	0.13 ± 0.01 (6)	7.9 ± 1.7	0.35 ± 0.08	1.16 ± 0.17	56 ± 8
Vitamin E supplemented	0.28 ± 0.04 (7)	4.6 ± 1.7	0.11 ± 0.02	0.87 ± 0.17	76 ± 4
P	<0.005	NS	<0.02	NS	NS

Data are means ± SEM of N = 5 individual animals per group except in the case of hepatic α-tocopherol content where numbers in parentheses indicate N values.

Table 2. Protective effect of vitamin E against protein sulphydryl group oxidation *in vitro*

Additions to incubation			Sulphydryl group content (nmol glutathione equivalents/mg protein)	
$ADP/Fe_2(SO_4)_3$	NADPH	α-Tocopheryl acetate (2 μM)	15 min incubation	60 min incubation
–	–	–	68 ± 2	67 ± 1
–	+	–	73 ± 6 (107)*	62 ± 2 (93)
+	+	–	36 ± 2 (53)†	39 ± 1 (58)†
+	+	+	54 ± 3 (79)‡	59 ± 5 (88)

Data are means ± SEM of triplicate determinations.

* Values in parentheses indicate percentage of control protein sulphydryl content (no additions to incubation).

Significant difference from control protein sulphydryl content: †$P < 0.001$, ‡$P < 0.05$.

quantitation. Cytochrome P450 IIC11 was quantitated in hepatic microsomes containing the active peroxidation system. At intervals after the initiation of peroxidation with NADPH, aliquots (0.2 mL) were removed to electrophoresis sample buffer (0.2 mL of buffer composed of 0.125 M Tris, pH 6.8, 20% glycerol, 4% sodium dodecyl sulphate, 0.004% Bromophenol blue and 10% 2-mercaptoethanol) and frozen immediately. Some reactions contained vitamin E acetate (2 μM) in addition to the lipid peroxidation system. At the conclusion of the experiment, the samples were boiled for 15 min, cooled and applied to polyacrylamide gels. Electrophoresis was conducted overnight, followed by electrophoretic transfer to nitrocellulose and immunoblotting for P450 IIC11 as described above.

Assay of microsomal protein sulphydryl groups. Total hepatic microsomal protein sulphydryl groups were estimated according to the procedure of Di Monte *et al.* [28] with the exception that 1 mg of microsomal protein was assayed. Glutathione was used as a reference standard for determination of sulphydryl group content.

Other assays. Serum sex hormones were determined by radioimmunoassay (CIS, Gif Sur Yvette, France). The testosterone assay exhibited 7.2 and 0.81% cross reactivity with dihydrotestosterone and androstenedione, respectively, and <0.04% for oestrogens, progestins and dihydroepiandrosterone. Cross reactivities of the oestradiol assay with oestrone and oestriol were 0.43% and 0.31%, respectively. Thyroid hormones (as plasma unbound triiodothyronine and thyroxine) were also quantified by radioimmunoassay (CIS).

α-Tocopherol was extracted from hepatic microsomes [29] and quantified by spectrofluorometry with authentic α-tocopherol (Sigma) as the standard [30].

Statistics. Differences between means were detected using the Student's *t*-test or Student–Newman–Keuls test following single factor analysis of variance.

RESULTS

Vitamin E supplementation and serum hormone levels in male rats

Supplementation of vitamin E intake (from 0.007 to 0.06% of diet) over a 6-week period resulted in an increase in hepatic microsomal α-tocopherol content over non-supplemented control values (0.28 ± 0.11 vs 0.13 ± 0.02 nmole/mg protein, $P < 0.005$, Table 1; by comparison, rats maintained on the standard commercial diet which contained 0.006% of vitamin E over the same period had α-tocopherol levels of 0.14 ± 0.04 nmol/mg protein). Supplementation did not influence the body weight, liver to body weight or testes to body weight ratios in male rats (not shown). Radioimmunoassay of serum hormones indicated that oestradiol was significantly decreased ($P < 0.02$) to 31% of vitamin E adequate control by modulation of dietary vitamin E intake (Table 1); testosterone, thyroxine and triiodothyronine were unchanged.

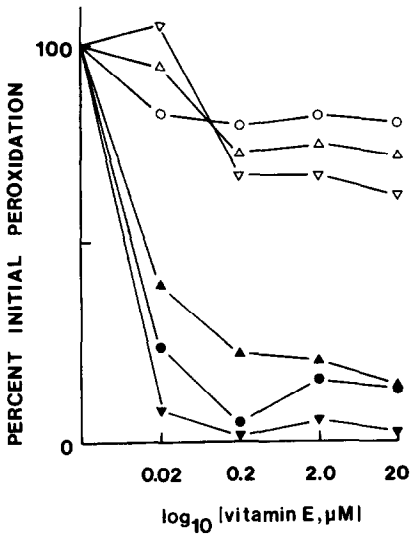

Fig. 1. Concentration-dependent effects of exogenous vitamin E acetate on lipid peroxidation (malondialdehyde formation) in control (vitamin E adequate; open symbols) and dietary vitamin E supplemented (closed symbols) rat liver microsomes. In the absence of exogenous vitamin E acetate, malondialdehyde formation was 1.88 ± 0.13 nmol/min/mg protein in control microsomes and 0.37 ± 0.27 in vitamin E-supplemented fractions.

Effect of dietary supplementation with vitamin E on microsomal protein sulphydryls and susceptibility to lipid peroxidation

The protein sulphydryl group content of microsomal fractions from control male rat liver was 68 ± 10 nmol of glutathione equivalents/mg protein (N = 6 per group). Vitamin E supplementation did not significantly affect this value (73 ± 6 nmol glutathione equivalents/mg protein). Consistent with other reports [2], α-tocopheryl acetate protected microsomal protein sulphydryl groups during peroxidation. From Table 2, 54 and 58% of microsomal sulphydryls were resistant to oxidation, after 15 and 60 min incubations, respectively, by the ferric sulphate–ADP–NADPH system. Protection of sulphydryl groups was afforded by vitamin E (2 μM; Table 2).

Dietary supplementation with (0.06%) vitamin E decreased markedly the susceptibility of microsomal lipids to peroxidation elicited by the $Fe_2(SO_4)_3$–ADP–NADPH system (Fig. 1). Thus, malondialdehyde formation during lipid peroxidation in vitamin E-supplemented rat liver microsomes was approximately 5-fold lower than in control fractions [0.37 ± 0.27 compared with 1.88 ± 0.13 nmol/min/mg protein ($P < 0.001$)]. The *in vitro* inclusion of exogenous vitamin E in the microsomal incubations decreased the extent of lipid peroxidation somewhat. In microsomal fractions from rats that received the dietary vitamin E supplementation, inclusion of additional exogenous vitamin E acetate (20 nM) in incubations *in vitro* decreased malondialdehyde production to 24 ± 9% of the rate measured in the absence of exogenous vitamin. From Fig. 2 it is evident that higher concentrations of exogenous vitamin E acetate (200 nM, 2 μM and 20 μM) decreased peroxidation further (to 9 ± 6, 14 ± 4 and 9 ± 4% of initial peroxidation, respectively). However, in control (vitamin E adequate) hepatic microsomes exogenous vitamin E acetate was much less effective in incubations *in vitro*. Approximately 30% inhibition of lipid peroxidation was observed when 200 nM to 20 μM vitamin E acetate was added to these microsomes (Fig. 2).

Effects of dietary vitamin E supplementation on hepatic microsomal cytochrome P450-mediated enzyme activities in the rat

Total microsomal cytochrome P450 was increased by 11% over control (vitamin E adequate) levels by dietary supplementation over the 6-week period of the study (1.32 ± 0.04 vs 1.19 ± 0.04 nmol/mg protein; $P < 0.05$; mean ± SEM; N = 6 per group; Table 3). NADPH-P450 reductase activity was also stimulated to 123% of control (Table 3). Regioselective androstenedione hydroxylation, which involves several P450s [19], was estimated and P450 IIC11-dependent steroid 16α-hydroxylase activity was increased in male rat liver by vitamin E supplementation (2.57 ± 0.13 vs 1.81 ± 0.16 nmol/mg protein/min; $P < 0.002$; Table 3), whereas formation of the 6β-, 7α- and 16α-hydroxysteroid metabolites was unchanged. Immunoquantitation of the male-specific P450s IIC11 and IIIA2 in these fractions was consistent with the findings from catalytic studies. Thus, elevated dietary vitamin E intake increased P450 IIC11 content by 44% over non-supplemented control (0.52 ± 0.05 vs 0.36 ± 0.07 nmol IIC11/mg protein, $P < 0.05$) whereas P450 IIIA2 content was unchanged. Following these findings the kinetic parameters of P450 IIC11-mediated testosterone 2α- and 16α-hydroxylation were estimated in hepatic microsomes from control and supplemented rat liver. In control hepatic microsomes the Michaelis constants (K_m) were found to be 12 ± 1 and 11 ± 1 μM (mean ± SEM, N = 3) for 2α- and 16α-hydroxylation, respectively, and were unchanged by dietary supplementation with vitamin E (13 ± 1 and 15 ± 1 μM, respectively; N = 3). However, a pronounced effect on the maximal reaction velocities (V_{max}) was noted with dietary supplementation (2.67 ± 0.13 vs 2.05 ± 0.06 nmol 2α-hydroxytestosterone formed/min/mg protein, $P < 0.02$; and 2.86 ± 0.14 vs 2.03 ± 0.08 nmol 16α-hydroxytestosterone formed/min/mg protein, $P < 0.01$). Finally, addition of α-tocopheryl acetate to microsomal incubations *in vitro* provided no evidence of binding to P450 and did not affect testosterone hydroxylase activities (data not shown). Thus, it is unlikely that alterations in microsomal vitamin E or the concentration of the vitamin at the P450 active site are responsible for the changes in P450 activity. Instead, the evidence is consistent with the notion that dietary vitamin E supplementation increases the microsomal content of P450 IIC11, the major steroid 2α- and 16α-hydroxylase of male rat liver.

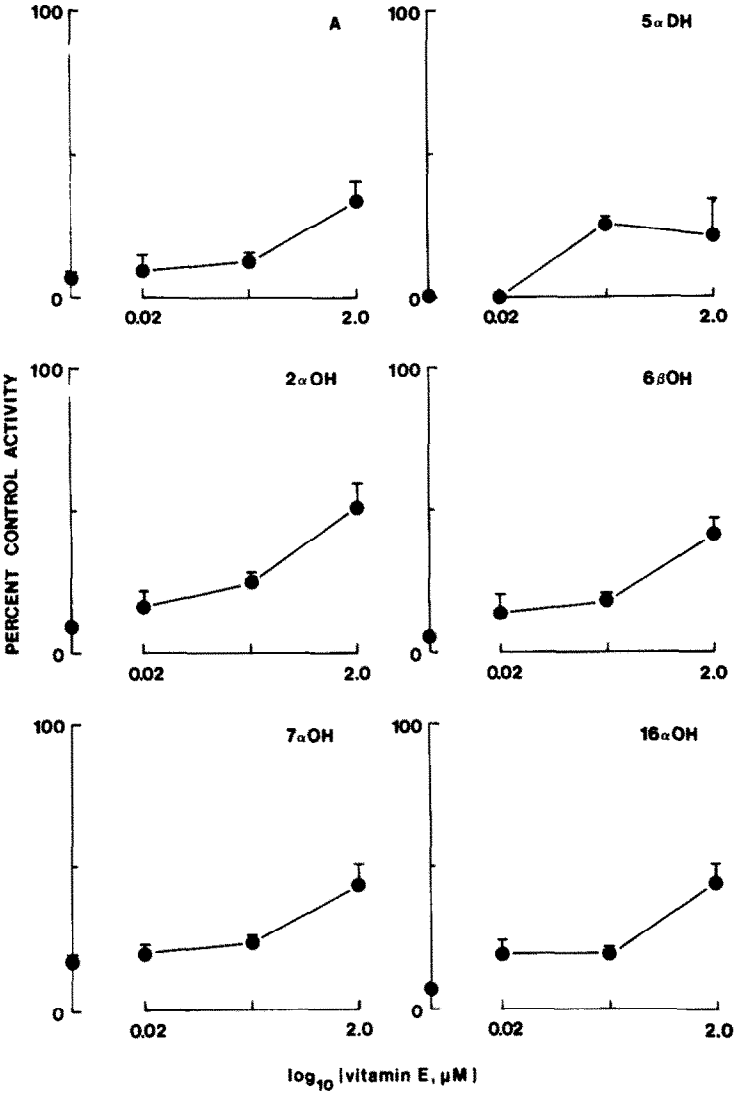

Fig. 2. Concentration-dependent effect of exogenous vitamin E acetate on *in vitro* lipid peroxidation-mediated decreases in microsomal testosterone metabolism. Data are means ± SEM (triplicate incubations at each point) and are presented as percentage of activity in the absence of peroxidation. The points on each axis are activities in the presence of peroxidation without exogenous vitamin E acetate. Key (control rates in parentheses): A, androstenedione (0.66 ± 0.19 nmol/min/mg protein); 5αDH, 5α-dihydrotestosterone (0.15 ± 0.02); 2αOH, 2α-hydroxytestosterone (0.57 ± 0.02); 6βOH, 6β-hydroxytestosterone (1.46 ± 0.10); 7αOH, 7α-hydroxytestosterone (0.15 ± 0.01) and 16αOH, 16α-hydroxytestosterone (0.79 ± 0.02).

In vitro *studies of the effects of exogenous vitamin E on microsomal P450-mediated oxidase activities during lipid peroxidation*

Under conditions where microsomal lipid peroxidation was catalysed extensively by the combination of ferric sulphate, ADP and NADPH, microsomal P450 content (determined spectrophotometrically by the ferrous–carbonyl complex) and P450-mediated steroid hydroxylations were decreased dramatically. Thus, total P450 content was decreased to 14% of control by activation of the lipid peroxidation system for 15 min (0.14 ± 0.04 nmol/mg protein compared with 1.02 ± 0.04; N = 3 assays each, mean ± SEM).

In agreement with the report of Kitada *et al.* [31], testosterone hydroxylations at the 2α-, 6β- and 16α-positions were very susceptible to deactivation during lipid peroxidation and were decreased to 5–9% of control (Fig. 2). 7α-Hydroxylation of the steroid was decreased to 16% of control and 5α-dihydrotestosterone formation mediated by the non-P450 enzyme Δ^4-3-oxosteroid 5α-oxidoreductase, was below detection. Inclusion of α-tocopheryl acetate in microsomal incubations partially protected

Table 3. P450, NADPH-P450 reductase and androstenedione hydroxylase activities in hepatic microsomes from male rats after dietary vitamin E manipulation

Diet	P450 (nmol/mg protein)	NADPH-P450 reductase (nmol/min/mg protein)	Hydroxyandrostenedione metabolite* (nmol/min/mg protein)			
			6β	7α	16α	16β
Vitamin E adequate	1.19 ± 0.04	195 ± 12	3.88 ± 0.19	0.25 ± 0.02	1.81 ± 0.16	0.29 ± 0.02
Vitamin E supplemented	1.32 ± 0.04	240 ± 16	4.12 ± 0.26	0.25 ± 0.01	2.57 ± 0.13	0.32 ± 0.01
P	<0.05	<0.05	NS†	NS	<0.005	NS
% Control	111	123	—	—	142	—

Data are means ± SEM of N = 6 animals per group, with the exception of the steroid hydroxylase data from the vitamin E-supplemented group where N = 7.

* Substrate (androstenedione) concentration in these experiments was 175 μM.

† NS, difference between mean values not significant.

against the loss of P450 activities (dose–response relationships shown in Fig. 2). Thus, restoration of P450 activities to 33–51% of those in the absence of peroxidation was obtained with the inclusion of 2 μM α-tocopheryl acetate.

Peroxidative loss of cytochrome P450 IIC11 apoprotein and reversal by exogenous vitamin E

In view of the data obtained for the effect of lipid peroxidation on total P450 and steroid hydroxylase activities, further studies investigated the effect of peroxidation-mediated changes in the apoprotein of P450 IIC11 (the microsomal testosterone 2α- and 16α-hydroxylase enzyme). Figure 3 presents the Western immunoblot analysis from which it is apparent that immunoreactive P450 IIC11 decreases with the length of incubation with the peroxidation system (lanes f–i). As is apparent from the figure, P450 IIC11 content after 15 min of incubation with the peroxidation system was decreased to 46% of the initial level. Figure 4 presents these findings quantitatively as a function of incubation time. In the absence of peroxidation, P450 IIC11 immunoreactivity was unchanged from the zero-time measurement (Fig. 3, lanes a–e and Fig. 4). Figure 3 also presents an immunoblot of P450 IIC11 obtained from an incubation that contained 2 mM exogenous vitamin E acetate in addition to an active peroxidation system (lane j). Importantly, vitamin E acetate completely protected P450 IIC11 from peroxidation-induced damage.

DISCUSSION

It seems clear from the present study that the *in vivo* and *in vitro* effects of vitamin E can now be considered quite separate. Dietary vitamin E manipulation influences P450 expression but, as seen from *in vitro* studies, the vitamin does not interact directly with the cytochrome at its active site. α-Tocopherol is an inhibitor of lipid peroxidation and sulphydryl group oxidation *in vitro*. However, neither of these mechanisms appear to constitute a straightforward explanation for the present data which show that the apoprotein of P450 IIC11, the major P450 in male rat liver, was preserved by exogenous vitamin E acetate even though the peroxidation-related decrease in total P450 was not reversed.

Inclusion of 2 μM exogenous vitamin E acetate in microsomal incubations *in vitro* resulted in a 30% decrease in lipid peroxidation and prevention of 55% of the oxidative loss of microsomal sulphydryl groups. Under identical conditions, it was found that P450-mediated steroid hydroxylase activities were also partially protected (to 33–51% of activities observed in the absence of peroxidation).

The mechanism of the loss of spectrally apparent P450 seems related to a specific destructive effect involving either loss of the P450 haem [32] or modification of the cysteine residue at the fifth axial position. This is a feasible explanation for two reasons. First, from this study, preliminary experiments established that vitamin E does not interact with P450 at its catalytic centre; spectral changes in the Soret region were not observed and the inclusion

Fig. 3. Western immunoblots of time-dependent changes in P450 IIC11 content in hepatic microsomes during peroxidation. Lane a is the blot of P450 IIC11 in zero-time incubations. Lanes b–e are blots of P450 IIC11 in microsomal incubations 2, 5, 10 and 15 min after NADPH addition and where ADP and $Fe_2(SO_4)_3$ were absent. Lanes f–i are blots of P450 IIC11 in incubations that included the complete peroxidation system. Lane j is the blot of P450 IIC11 from a 15-min incubation that included the complete peroxidation system and 2 μM exogenous vitamin E acetate.

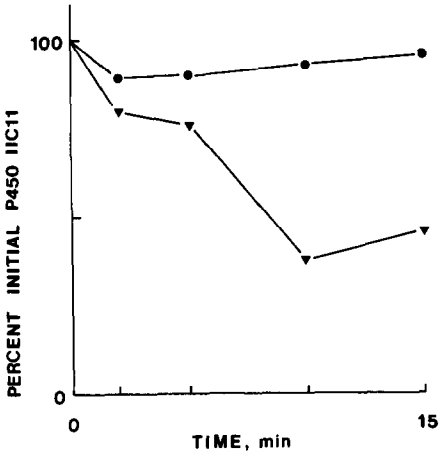

Fig. 4. Graph of changes in microsomal P450 IIC11 content in the presence (▼) and absence (●) of lipid peroxidation. Data at each time point are means of estimates derived from two separate incubations; the data varied by less than 15% from the stated values.

of α-tocopheryl acetate in microsomal incubations *in vitro* at concentrations of up to 100 μM had no effect on steroid hydroxylase activities. Second, vitamine acetate only partially prevented the loss of P450 activities during microsomal peroxidation, whilst protecting the apoprotein completely, which suggests that the vitamin has limited access to the destructive species.

It is clear that vitamin E affords protection against peroxidation-mediated loss of P450 apoprotein. A previous study also described decreases in the microsomal content of certain P450s during peroxidation [31]. That this occurs under *in vitro* conditions suggests that the membrane anchors of susceptible P450s are degraded. It has been proposed that transmembrane sequences of 20–30 amino acid residues anchor P450s to the endoplasmic reticulum [33]. Peroxidative damage to this segment would be expected to disorient the P450, perhaps resulting in complete loss of the protein from its membrane location. Microsomes from rats that received *in vivo* supplementation with vitamin E were more resistant to peroxidative damage *in vitro*. Furthermore, an interesting point that emerged from the present study was that *in vitro* addition of vitamin E acetate was more effective in preventing lipid peroxidation in microsomes from rats that received the dietary vitamin E supplementation. It is possible that this effect is related to differences between *in vitro* and *in vivo* modes of the incorporation of tocopherols into the microsomal membrane.

The present study evaluated the effects of dietary supplementation with α-tocopheryl acetate on the catalytic function of specific P450s. The activities of three constitutive P450s important in steroid hydroxylation were assessed and an increase in the activity of the major P450 enzyme IIC11 was noted (reflected by increased rates of microsomal testosterone 2α- and 16α-hydroxylation). No changes were observed in androstenedione 6β- (P450 IIIA2-mediated) or 7α-hydroxylation (P450 IIA1- and, to a lesser extent, P450 IIA2-mediated). Although a change in the activity of the microsomal NADPH-P450 reductase was observed, immunoquantitation experiments confirmed an increase in microsomal P450 IIC11 content. Therefore, direct evidence was obtained that P450 IIC11 is influenced by dietary vitamin E and that altered rates of substrate oxidation are not due to a change in microsomal P450-reductase concentration.

The present data indicate that dietary vitamin E supplementation upregulates P450IIC11. To date few treatments have been described that result in the upregulation of this enzyme in male rat liver. The close relationship between hormonal status and P450 IIC11 expression necessitated the serum hormone measurements in Table 1. Oestradiol was decreased in serum of vitamin E-supplemented animals but testosterone, thyroxine and triiodothyronine were unchanged. Since down-regulation of P450 IIC11 by oestradiol has been described [34], it is conceivable that vitamin E supplementation may exert a derepressive effect of the female hormone on IIC11 in normal male rat liver. Further studies are required to assess possible mechanisms for such an effect.

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