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Authors

BONDY, STEPHEN C
GUO, SHIRLEY X
ADAMS, JAMES D

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PREVENTION OF ETHANOL-INDUCED CHANGES IN REACTIVE OXYGEN PARAMETERS BY α -TOCOPHEROL

STEPHEN C. BONDY*, SHIRLEY X. GUO and JAMES D. ADAMS¹

Department of Community and Environmental Medicine, Irvine Occupational Health Center, University of California, Irvine, Irvine, CA 92717-1825 and ¹Department of Molecular Pharmacology and Toxicology, University of Southern California, Los Angeles, CA 90033, USA

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Abstract — Rats were given a 200 mg/kg body weight daily dose of α -tocopherol by i.p. injection for 15 days. This resulted in elevated levels of glutathione in both liver and brain, and in a reduced hepatic rate of generation of reactive oxygen species. The depression of hepatic and cerebral glutathione levels in ethanol-consuming rats was prevented by simultaneous treatment with α -tocopherol. Other putative indices of hepatic pro-oxidant events, namely levels of mixed function oxidase and proteolytic activity, were elevated by α -tocopherol both in the presence and absence of ethanol. In addition, levels of enzymes especially susceptible to oxidative degradation, glutamine synthetase and creatine kinase, were depressed in the liver following treatment with ethanol or α -tocopherol. Parameters rapidly responsive to oxidative changes revealed the antioxidant property of α -tocopherol, while protein-based indices reflecting more extended events suggested a pro-oxidant effect of this vitamin. Results suggest that high levels of α -tocopherol can simultaneously lead to a more reduced intracellular environment and yet to localized evidence of enhanced oxidative events.

INTRODUCTION

There is accumulating evidence that some of the deleterious effects of ethanol can be attributed to the induction of metabolic processes which lead to the generation of excessive levels of reactive oxygen species (ROS). Such events are more unequivocally associated with ethanol-induced liver damage (Dicker and Cederbaum, 1988; Cederbaum, 1989; Reinke and McCay, 1991; Bondy, 1992), but there are also data suggesting a parallel sequence of events within nervous tissue (Nordmann, 1987). There are reports of α -tocopherol depletion in ethanol-consuming animals (Hagen *et al.*, 1989), and in human alcoholics (Bell *et al.*, 1992), and also of the beneficial effects of supplementation with this vitamin during ethanol consumption (Nadiger *et al.*, 1988). It has therefore been proposed that antioxidant vitamins may mitigate the severity of ethanol toxicity. As a preliminary step toward investigating this issue, we have examined the effect of α -tocopherol dosing upon cerebral and hepatic indices of oxidant activity.

Results obtained suggest that high levels of α -tocopherol may be accompanied by metabolic changes implying that both antioxidant and pro-oxidant events may be initiated by α -tocopherol. Nevertheless α -tocopherol appears to prevent several changes in parameters of oxidative stress affected by ethanol. α -Tocopherol is able to enter the central nervous system and effect detectable biochemical changes in a relatively short period.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA, USA) weighing 150–175 g were utilized. Rats were housed four per cage with wood chip bedding and maintained on a 12 h light/dark cycle in a temperature-controlled ($20 \pm 1^\circ\text{C}$) room. Food (Purina Laboratory Chow, St Louis, MO, USA) and water were provided *ad libitum* until the start of the feeding protocol.

Ethanol and α -tocopherol treatment

α -Tocopherol succinate was administered daily for 15 days to two groups of rats by intraperitoneal

*Author to whom correspondence should be addressed

injection in corn oil (200 mg/kg body wt). Control groups received an identical volume of corn oil alone (1 ml/kg body wt). An all-liquid nutrient diet (Ross Laboratories, Columbus, OH, USA) was supplied to all rats (Bondy and Pearson, 1993). All rats received 88% Ensure, 12% water (v/v) for 3 days. Then half of the rats received 88% Ensure-7% water-5% ethanol (v/v) for 3 further days. Fresh solutions were made up daily. At this time, the ethanol proportion was raised to 7% while water was reduced to 5%. Animals were decapitated after a total of 12 ethanol-drinking days. The fluid intake of the control group, drinking the liquid nutrient alone, was limited to 120% of the volume drunk on the previous day by the group receiving ethanol-containing liquid diet. This was isocaloric with the original ethanol-contained nutrient. Rat weights were monitored and recorded every second day, together with fluid consumption of each group.

Tissue preparation

Rats were decapitated, the brains were excised quickly on ice and the cerebrocortex was dissected out. The liver was removed and frozen at -70°C . All tissues were placed into screw-capped micro-centrifuge tubes, stored at -20°C for 24 h and subsequently stored at -70°C until preparation. The relatively slow freezing rate was intended to maintain the integrity of subcellular structures (Dodd *et al.*, 1981). Each tissue was weighed and homogenized in 10 vol of 0.32 M sucrose and centrifuged at 1800 g for 10 min. The resulting supernatant fraction was then centrifuged at 31 500 g for 10 min to yield the crude hepatic or cerebral mitochondrial pellet (P2) and the supernatant (S2). The P2 pellet was resuspended in HEPES buffer to a concentration of 0.1 g-eq/ml. The final protein content of P2 and S2 suspensions was 1.6–3.1 mg/ml. The composition of the HEPES buffer was (mM): NaCl, 120; KCl, 2.5; NaH_2PO_4 , 1.2; MgCl_2 , 0.1; NaHCO_3 , 5.0; glucose, 6.0; CaCl_2 , 1.0; and HEPES, 10; pH 7.4.

Assay of α -tocopherol

α -Tocopherol was measured after homogenization of 50 mg tissue samples in a mixture of 0.5 ml of 1% (w/v) sodium ascorbate in ethanol and 0.5 ml of 0.14 M NaCl. This was then shaken with 2 ml of *n*-hexane and centrifuged (5000 g, 5 min). The supernatant hexane layer was removed and

lyophilized under nitrogen. The residue was reconstituted in methanol and injected into a high-performance liquid chromatography system, using methanol as the mobile phase. α -Tocopherol was then determined in the eluate with a fluorescence detector (excitation: 230 nm, emission: 340 nm).

Glutamine synthetase

This soluble enzyme was assayed as γ -glutamyl transferase activity by incubation (30 min, 37°C) of 0.1 ml of S2 preparation together with (mM) L-glutamine (50), hydroxylamine (75), NaADP (0.5), MnCl_2 (0.2), HCl (50), sodium arsenate (25), in a final volume of 1 ml. γ -Glutamyl hydroxamate formed could then be quantitated after centrifugation (5000 g, 5 min) by spectrophotometric assay of the coloured product formed with acidified FeCl_3 (Rowe *et al.*, 1970). A standard curve was concurrently generated, with γ -glutamyl hydroxamate. The iron complex of 1 mmol/ml of this compound gave an absorbance of 0.340 at 535 nm. When the effect of various agents on enzyme activity was studied, a 30 min preincubation of the S2 fraction with these chemicals preceded enzyme assay.

Creatine kinase

This enzyme was assayed by determination of ATP formed from phosphocreatine in the presence of ADP. ATP was then permitted to phosphorylate glucose in the presence of hexokinase, and the resulting glucose 6-phosphate could convert NADP^+ to NADPH following addition of glucose 6-phosphate dehydrogenase (Szasz *et al.*, 1976). NADPH was then read spectrophotometrically at 340 nm. Incubation was at pH 6.7 for 2 min at 22°C in a medium containing (mM) creatine phosphate (30), ADP (2), AMP (5), NAD^+ (2), MgCl_2 (10), glucose (20), EDTA (2), Tris-HCl, (50), hexokinase 3000 U/l, glucose 6-phosphate dehydrogenase, 2000 U/l. *N*-Acetyl cysteine 20 mM was also present in order to activate creatine kinase (Szasz *et al.*, 1976).

Assay for oxygen reactive species formation

Reactive oxygen species were assayed using 2',7'-dichlorofluorescein diacetate (DCFH-DA), which is de-esterified within cells to the ionized free acid, DCFH. This is trapped within cells and thus accumulated (Bass *et al.*, 1983). DCFH is

capable of being oxidized to the fluorescent 2',7'-dichlorofluorescein by reactive oxygen. The utility of this probe in isolated subcellular cerebral systems has been documented (LeBel and Bondy, 1990). Fifty μ l of P2 suspension were incubated with 5 μ M DCFH-DA (added from a stock solution of 1.25 mM in ethanol) in a final volume of 2 ml 40 mM Tris-HCl, pH 7.4, at 37°C for 15 min. After this loading with DCFH-DA, the fractions were incubated for a further 60 min in the presence of various enzymes or pharmacological inhibitors. At the beginning and at the end of incubation, fluorescence was monitored on a Perkin-Elmer Spectrofluorimeter, with excitation wavelength at 488 nm (bandwidth 5 nm), and emission wavelength 525 nm (bandwidth 20 nm).

Determination of tissue glutathione

Reduced glutathione (GSH) levels were determined using a modification of the method of Shrieve *et al.* (1988). The principle of the assay is that monochlorobimane (mBCl), a non-fluorescent compound, reacts with glutathione to form a fluorescent adduct. It has been shown that there is very little reaction between mBCl and protein sulphhydryl groups (Rice *et al.*, 1986). mBCl was dissolved in ethanol to a concentration of 5 mM and stored at -10°C in the dark. mBCl was added to 2 ml of an S2 suspension to a final concentration of 20 μ M, after which the suspension was incubated for 15 min at 37°C. The fluorescence of the supernatant was read on a Perkin-Elmer Spectrophotofluorimeter at an excitation wavelength of 395 nm and an emission wavelength of 470 nm. The tissue GSH concentration was determined using a GSH standard curve. This determination was performed in the presence of 0.1 unit/ml of liver glutathione transferase in order to accelerate the formation of the fluorescent adduct derivative.

Assay for proteolytic activity

Serine protease activity was measured by a method utilizing the cleavage of a synthetic peptide, whose hydrolytic cleavage leads to the appearance of rhodamine, which is quantified by fluorescent assay (Leytus *et al.*, 1983). Bis (*N*-benzyloxycarbonyl-L-arginamido) rhodamine [(Cbz-Arg-NH₂)₂-rhodamine], dissolved in ethanol, was added at a final concentration of 10 μ M, to 2 ml of S2 of suspension in Tris-HCl buffer.

The appearance of rhodamine over a 1 h incubation at 37°C was determined fluorometrically (excitation: 492 nm, emission: 523 nm).

Mixed function oxidase

Cytochrome P-450 enzyme activity was determined using the method described of Buters *et al.* (1993). EFC (7-ethoxy-4-trifluoromethylcoumarin) (4 mM) in 5 μ l of dimethylsulphoxide, was added to 2 ml of Tris-HCl containing 0.2 mM NADPH and 0.2 ml S2 suspension. The mixture was incubated at 37°C for 1 h and the fluorescence of the O-de-ethylated product, 7-hydroxy-4-trifluoromethylcoumarin (HFC) was determined at 510 nm using an excitation wavelength of 410 nm. This probe is relatively non-specific and allowed assay of a broad range of mixed function oxidases.

Materials

DCFH-DA, 7-ethoxy-4-trifluoromethylcoumarin (EFC), bis (*N*-benzyloxycarbonyl-L-arginamido) rhodamine, monochlorobimane (mBCl) were purchased from Molecular Probes, Inc. (Eugene, OR, USA), whereas DCF required for calibration was obtained from Polysciences, Inc. (Warrington, PA, USA). Protein assay dye was from Bio-Rad Laboratories (Hercules, CA, USA). All other chemicals were obtained from Sigma Chemical Co. (St Louis, MO, USA).

Protein determination

Protein concentration was assayed using the method of Bradford (1976).

Statistical analyses

Differences between groups were assessed by one-way analysis of variance (ANOVA) followed by Fisher's least significant difference test. The acceptance level of significance was $P < 0.05$ using a two-tailed distribution. Each value presented was obtained from 6-8 individual rats.

RESULTS

The addition of α -tocopherol to the diet led to a significantly higher concentration of this vitamin in both hepatic and cerebral tissues. This increase was much more pronounced in the liver. These values were not significantly modulated in the corresponding groups of animals that were treated with ethanol (Table 1). Rates of ethanol consump-

Table 1. Cerebral and hepatic levels of α -tocopherol in untreated and ethanol-exposed rats

Group	α -Tocopherol ($\mu\text{g/g}$ wet wt)	
	Brain	Liver
Control untreated	4.17 \pm 0.23	3.8 \pm 1.4
Ethanol-treated	5.08 \pm 0.09	4.0 \pm 1.7
α -Tocopherol-supplemented	5.74 \pm 0.32*	9.5 \pm 1.6*
α -Tocopherol-supplemented and ethanol-treated	6.07 \pm 0.22*	12.8 \pm 3.7*

Values are means \pm SEM.

*Differs significantly from the corresponding values for rats not receiving α -tocopherol supplementation.

tion over the preceding 12 days were similar in the two ethanol-consuming groups, namely 13.8 ± 1.9 and 9.6 ± 1.7 g of ethanol/kg body weight/day for the ethanol and ethanol + α -tocopherol groups respectively.

Both groups of rats receiving α -tocopherol exhibited fibrosis of the liver and had a peritoneal cavity containing ascites fluid, suggesting that at the dose used, this vitamin was hepatotoxic (results not shown).

Following administration of α -tocopherol, glutathione levels in both brain and liver were elevated, whereas ethanol treatment depressed glutathione in both tissues. When both agents were administered together, glutathione levels did not differ significantly from those of untreated rats (Fig. 1).

The rate of generation of DCF from DCFH was diminished in the hepatic P2 fraction after α -tocopherol treatment either in the presence or absence of ethanol (Fig. 2). This implied a reduced

rate of ROS generation in vitamin-treated rats. However, levels of glutamine synthetase were reduced in liver, by either ethanol and α -tocopherol separately or in combination, suggesting oxidant-initiated degradation may result from either treatment (Fig. 3). This concept was supported by the finding that another enzyme that is especially susceptible to oxidative damage, creatine kinase, was also depressed by either ethanol or α -tocopherol or both agents in combination, in a parallel manner (Fig. 3).

The reduction of hepatic glutathione induced by ethanol alone despite no significant changes in the rate of DCFH oxidation is concordant with earlier work of ours (Bondy and Guo, 1994). Cerebral levels of DCF production, glutamine synthetase or creatine kinase, were unaffected by α -tocopherol dosing (data not shown).

The α -tocopherol regimen resulted in an elevated level of proteolytic activity in the hepatic S2 fraction, and this was maintained in animals

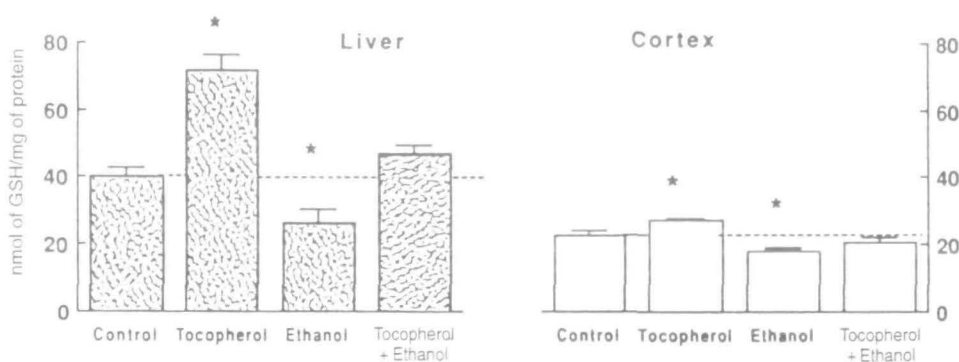


Fig. 1. Cytosolic glutathione (GSH) content in cerebral cortex and liver from ethanol- or α -tocopherol-treated rats. Each datum point represents the mean of values derived from six individual animals \pm SEM. *Level differs significantly from the corresponding control ($P < 0.05$, Student's *t*-test after analysis of variance and Fisher's least significant difference).

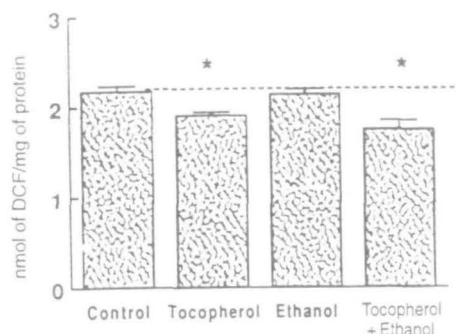


Fig. 2. Rates of reactive oxygen species (ROS) formation in hepatic P2 fraction from rats treated with ethanol or α -tocopherol.

Each datum point represents the mean of values derived from six individual animals \pm SEM. *Level differs significantly from the corresponding control ($P < 0.05$, Student's *t*-test after analysis of variance and Fisher's least significant difference).

treated with both ethanol and α -tocopherol (Fig. 4). Exposure to ethanol alone led to a reduction in hepatic protease activity. Changes in cerebral proteolytic rates in response to these treatments were less than 10% (data not shown).

The finding that livers of ethanol-treated animals exhibited a major increase in proteolytic activity, and decline in glutamine synthetase levels, was compatible with an elevation of cytochrome *P*-450 oxidase-promoted ethanol catabolism leading to excessive pro-oxidant activity. Hepatic levels of mixed function oxidase were therefore examined. These were greatly enhanced

in the livers of α -tocopherol treated rats, whether or not their diet included ethanol (Fig. 5). Ethanol alone depressed overall cytochrome *P*-450 oxidase activity. Cerebral levels of cytochrome *P*-450 oxidases were $<1\%$ of hepatic values and therefore difficult to determine with accuracy.

DISCUSSION

Several pieces of evidence reported here suggest that the high level of α -tocopherol used led to oxidative stress in the liver. This antioxidant vitamin induced mixed-function oxidases in the liver. Such an induction suggests that, if present in sufficient amounts, this vitamin may act as a substrate for mixed function oxidases. The ability of antioxidants to protect mono-oxygenases from degradation (Nagababu and Lakshmiiah, 1994) may paradoxically result in the promotion of pro-oxidant events. This may then lead to an enhancement of oxidative degradation of proteins (Dicker and Cederbaum, 1988), that we have also observed here.

Under some circumstances, α -tocopherol can accelerate oxidative events, since some of the catabolic products of this vitamin such as the α -tocopheroxyl radical have pro-oxidant properties (Maiorino *et al.*, 1993; Iwatsuki *et al.*, 1995), and this may relate to the induction of oxidases described here. The metabolite α -tocopherol quinone, which is also capable of redox cycling, is normally present in very low amounts intracellularly, but may be elevated at high levels of α -

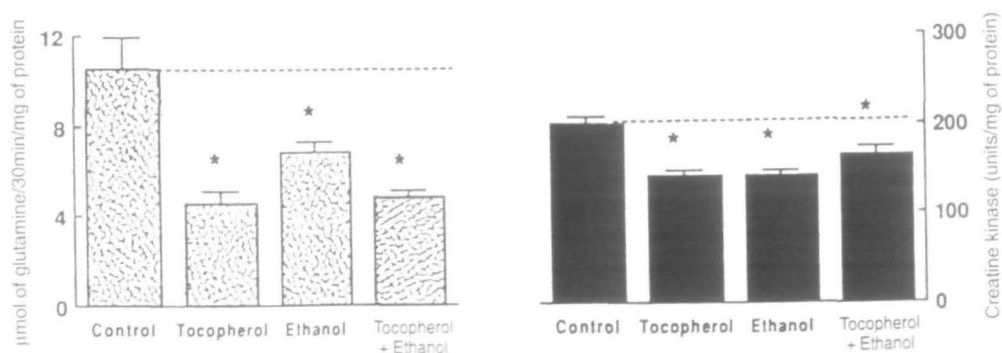


Fig. 3. Hepatic levels of glutamine synthetase and creatine kinase in post-mitochondrial supernatants of ethanol-consuming or α -tocopherol-treated rats.

Each datum point represents the mean of values derived from 6 individual animals \pm SEM. *Level differs significantly from the corresponding control ($P < 0.05$, Student's *t*-test after analysis of variance and Fisher's least significant difference).

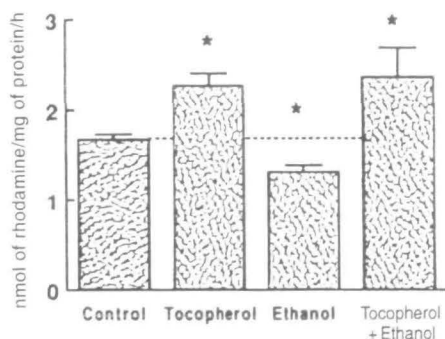


Fig. 4. Proteolysis in post-mitochondrial supernatants from livers of ethanol- or α -tocopherol-treated rats.

Values represent rate of hydrolysis of bis-(*N*-benzyloxy-carbonyl-L-arginamido) rhodamine [(Cbz-Arg-NH₂)₂rhodamine]. Each datum point is the mean of values derived from six individual animals \pm SEM. *Level differs significantly from the corresponding control ($P < 0.05$, Student's *t*-test after analysis of variance and Fisher's least significant difference).

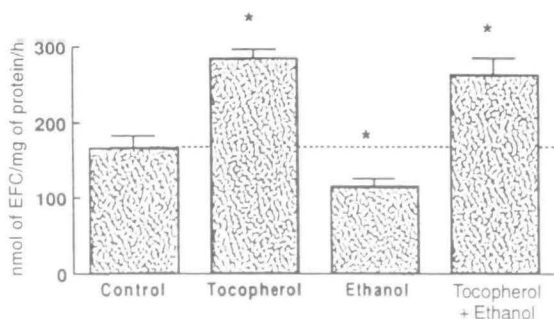


Fig. 5. Hepatic mixed function oxidase activity in S2 fractions from rats treated with ethanol or α -tocopherol.

Values represent rate of oxidation of EFC. Each datum point is the mean of values derived from six individual animals \pm SEM. *Level differs significantly from the corresponding control ($P < 0.05$, Student's *t*-test after analysis

tocopherol intake.

Whether α -tocopherol acts as a pro- or anti-oxidant may therefore depend on dose- and time-related determinants, and may differ within various cellular compartments.

Mixed-function oxidases are capable of producing ROS even in the absence of substrate (Naqui and Chance, 1986; Bondy and Naderi, 1994). Cytochrome *P*-450 2E1 is especially potent in generation of ROS under these conditions

(Ekstrom and Ingelman-Sundberg, 1989). A major induction of such isozymes could be expected to raise cytosolic rates of ROS production. The major ethanol-catabolizing microsomal isoenzyme is cytochrome *P*-450 2E1 (Reinke and Moyer, 1985). Around 60% of the activity assayed by EFC, the substrate used in this study, may be due to this isozyme (Buters *et al.*, 1993). However, since ethanol alone depressed rather than elevated mixed-function oxidases, the affinity of the EFC substrate for the *P*-450 2E1 iso-enzyme is not likely to be as specific as previously claimed (Buters *et al.*, 1993). It may be that a compensatory depression of other cytochrome *P*-450 species takes place concomitantly with ethanol-effected induction of cytochrome *P*-450 2E1. Our results are in accord with an earlier report of the failure of ethanol treatment to accelerate oxidation of 7-ethoxy-4-trifluoromethylcoumarin (Deluca *et al.*, 1989).

Glutamine synthetase and creatine kinase are readily inactivated by active oxygen species in a site-specific manner (Levine *et al.*, 1981; Fucci *et al.*, 1983; Rivett, 1985; Schor, 1988). Therefore the depressed levels of these enzymes observed in rats treated with α -tocopherol alone suggest the presence of excess ROS. Since proteases have been reported to be elevated consequent to an overabundance of oxidatively damaged proteins, induction of these enzymes has been taken as evidence of oxidative stress (Davies *et al.*, 1987; Dean, 1987; Vince and Dean, 1987; Davies, 1993). Thus the enhanced rates of protein degradation in the liver α -tocopherol-treated rats further suggest elevated rates of generation of ROS. Ethanol has previously been found to inhibit proteolysis in the liver and to lead to an accumulation of excess protein in this organ (Donohue *et al.*, 1989; Poso and Hirsimaki, 1991). In the present work, the cytosolic fraction of liver from subchronically treated rats also showed a significant reduction of proteolytic activity (Fig. 4), similar in magnitude to those of the earlier reports. It may be that events unrelated to increased ROS production predominate in inhibiting proteases in the liver after ethanol treatment.

Both depressed and elevated levels of glutathione have been taken to reflect a response to enhanced levels of pro-oxidant activity (Adams *et al.*, 1993). We have found hepatic glutathione

levels to respond to ethanol treatment in a biphasic manner, being initially elevated and then depressed relative to basal values (Bondy and Guo, 1994).

The elevation of proteolytic activity and inhibition of activities of oxidant-susceptible enzymes imply the induction of hepatic elevated pro-oxidant levels by α -tocopherol. These events were not apparent in brain tissue, perhaps because the elevation of α -tocopherol levels in this organ of treated animals was only $\sim 38\%$ above basal values while the corresponding elevation in the livers was 250% (Table 1).

While most evidence suggests that α -tocopherol in the doses used here elevates hepatic free radical production, the depressed rate of DCFH oxidation is in conflict with this. There are previous examples wherein oxidant status as judged by indices of an assay reflecting short-term status is not concordant with protein-based indices reflecting the sum of events over a more extended period (LeBel and Bondy, 1991).

Despite the bidirectional changes in oxidant indices effected by α -tocopherol, this agent appears to reverse several of various metabolic consequences of ethanol consumption in both brain and liver. However, excessive levels of this vitamin can be hepatotoxic and the generation of oxidative stress may be a factor underlying this. Similar protective effects against ethanol have been demonstrated following dietary supplementation with lesser amounts of this vitamin administered (1000 ppm, Bondy *et al.*, 1996). In this case, basal levels of the parameters assayed were unaltered by the high α -tocopherol diet. There is clearly an optimal level where antioxidant protection may mitigate against some of the effects of ethanol but exceeding this may cause a range of unintended metabolic consequences.

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