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Dietary α-tocopherol content modulates responses to moderate ethanol consumption

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Abstract

Rats were fed with diets containing differing amounts of α-tocopherol for 21 days. For the latter 14 days of this period, one half of the rats also received ethanol (7% v/v) in the drinking water. Treatments did not alter the rate of weight gain between groups. Hepatic glutathione levels were depressed by ethanol treatment in rats receiving diets deficient in α-tocopherol or containing normal levels of the vitamin (50 ppm). However, this depression was not found in rats maintained on a high α-tocopherol diet (1000 ppm). The high α-tocopherol diet also prevented the ethanol-induced inhibition of proteolytic activity within the liver. A dose-dependent reduction of rates of hepatic generation of reactive oxygen species was effected by this vitamin. Within the central nervous system, the only region showing an ethanol-induced lowering of glutathione levels, was the midbrain of rats receiving the α-tocopherol deficient diet.

Keywords: Ethanol; α-Tocopherol; Oxidative stress; Reactive oxygen

1. Introduction

Some of the harmful effects of ethanol have been attributed to the induction of metabolic processes which lead to the generation of excessive levels of reactive oxygen species (ROS). Such changes have been invoked as underlying ethanol-induced liver damage (Cederbaum, 1989; Reinke and McCay, 1991; Bondy, 1992) but there are also data suggesting a parallel sequence of events within nervous tissue (Nordmann, 1987). α-Tocopherol levels may be depressed in experimental animals exposed to ethanol (Hagen et al., 1989), and in human alcoholism (Bell et al., 1992). Beneficial effects of supplementation with this vitamin during ethanol consumption have also been reported(Nadiger et al., 1988). It may be that antioxidant vitamins have therapeutic value in reducing the severity of ethanol toxicity. We have previously found a protective effect of high doses of injected α-tocopherol on animals consuming large amounts of ethanol (Bondy and Guo, 1995). The present work investigated the effect of more modest dietary treatment with this vitamin. The responses to treatment of two indices of oxidative stress were evaluated, and also hepatic rates of proteolysis which have been found to be depressed by ethanol (Donohue et al., 1989; Bondy and Guo, 1995). In addition, a relatively lower ethanol drinking regimen was employed that did not affect normal rates of weight accretion in experimental animals. The protective effect of α-tocopherol was maintained under these conditions which more closely approximate potential human exposures.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 125–150 g were utilized. Rats were housed three per cage with wood chip bedding and maintained on a 12 h light/dark cycle in a temperature controlled (20 ± 1°C) room. Food and water were provided ad lib until the start of the feeding protocol.

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2.2. Ethanol and α-tocopherol treatment

Rats received one of three diets ad lib differing by their content of α-tocopherol acetate, for 21 days. These contained 0, 50, 1000 ppm of the vitamin respectively and were prepared by Dyets, Bethlehem, PA. 1 week after commencing this diet, half of the rats under each dietary regimen were retained on normal drinking water while the other half received water containing 5% (v/v) ethanol for 2 days, followed by 7% (v/v) ethanol for the remaining 12 days. Six animals were in each final group. Body weights were monitored and recorded every second day, together with fluid consumption of each group.

2.3. Tissue preparation

Rats were decapitated and liver and brains were excised. Brain regions (cerebrocortex, midbrain, striatum and cerebellum) were then dissected out on dry ice. All tissues were placed into screw-capped microcentrifuge tubes, stored at $-70°C$ until preparation. Each tissue was weighed and homogenized in 10 vols. of 0.32 M sucrose and centrifuged at $1800 \times g$ for 10 min. The resulting supernatant fraction was then centrifuged at $31\,500 \times g$ 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 2.1–14.5 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.

2.4. Assay of α-tocopherol

Hepatic α-tocopherol was measured after homogenization of unfractionated 50 mg tissue samples in a mixture of 0.5 ml 1% (w/v) sodium ascorbate in ethanol and 0.5 ml 0.14 M NaCl. This was then shaken with 2 ml hexane and centrifuged $(5000 \times g,$ 5 min). The supernatant hexane layer was removed and lyophilized under nitrogen. The residue was reconstituted in methanol and injected into an octadecyl silyl reverse phase HPLC system, using methanol as the mobile phase and a flow rate of 1 ml/ml. α-Tocopherol was then determined in the eluate with a fluorescence detector (excitation: 230 nm, emission: 340 nm) using δ-tocopherol as an internal standard.

2.5. Assay for oxygen reactive species formation

Reactive oxygen species (ROS) were assayed using 2',7'-dichlorofluorescein diacetate (DCFH-DA), which is de-esterified within cells to the ionized free acid, DCFH, where it is accumulated (Bass et al., 1983). DCFH is capable of being oxidized to the fluorescent 2',7'-dichlorofluorescein by reactive oxygen. The oxidation of DCFH is

by short-lived ROS. Despite the fact that the terminal oxidant species have not been definitively characterized, DCFH is a useful probe as an overall index in the study of oxidative stress in toxicological phenomena (LeBel et al., 1992). 50 μl P2 suspension was incubated with 5 μM 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. At the beginning and at the end of 60 min incubation, fluorescence was monitored on a Perkin-Elmer spectrofluorometer, with excitation wavelength at 488 nm, and emission wavelength 525 nm.

2.6. Determination of tissue glutathione

Glutathione (GSH) levels were determined within S2 fractions using a modification of the method of Shrieve et al. (1988). The principle behind the assay is that monochlorobimane (mBCl), a nonfluorescent compound, reacts with glutathione to form a fluorescent adduct. This method is rather specific since mBCl reacts with protein thiols at a much lower rate than it conjugates with glutathione in the presence of glutathione S-transferase (Shrieve et al., 1988). 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 a S2 suspension to a final concentration of 10 μM, after which the suspension was incubated for 15 min at 37°C. The fluorescence of the supernatant was spectrophotometrically determined at an excitation wavelength 395 nm and an emission wavelength 470 nm. The tissue GSH concentration was calculated using a GSH standard curve.

2.7. Assay for proteolytic activity

Serine protease activity within S2 fractions was measured by a method utilizing a synthetic peptide, whose hydrolytic cleavage leads to the appearance of rhodamine, which is quantitated by fluorescent assay (Leytus et al., 1983). Bis-(N-benzyloxycarbonyl-L-arginamido) rhodamine $((Cbz-Arg-NH_2)_2$ rhodamine), dissolved in ethanol, was added at a final concentration of 10 μM, to 2 ml 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).

2.8. Materials

DCFH-DA, $(Cbz-Arg-NH_2)_2$ rhodamine, and mBCl were purchased from Molecular Probes (Eugene, OR). while DCF required for calibration was obtained from Polysciences (Warrington, PA). Protein assay dye was from Bio-Rad Laboratories (Hercules, CA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

2.9. Protein determination

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

2.10. Statistical analyses

Differences between groups were assessed by one-way Analysis of Variance 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.

3. Results

The growth rate of rats in each of the four groups did not differ. Rats weighed 141 ± 3 g at the start of treatment and 201 ± 4 g at the termination of the study. Ethanol consumption of the ethanol-drinking groups was 4.2 ± 0.3 g/kg body weight/day/rat and each rat ate 13–17 g of diet daily. Treatment with the α-tocopherol deficient diet led to hepatic levels of this vitamin that were significantly below those in the corresponding group receiving a standard amount of α-tocopherol (50 ppm) in the diet. Hepatic α-tocopherol content was lowest in the ethanol consuming group fed a deficient diet. With the high α-tocopherol diet (1000 ppm), liver concentration of this vitamin was maximal (Fig. 1).
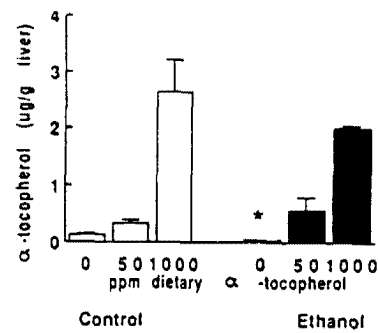

Fig. 1. Hepatic levels of α-tocopherol in rats receiving several diets, in the presence and absence of ethanol consumption. Each value represents the mean ± SE derived from five individual rats. *, differs from the corresponding group not consuming ethanol ($p > 0.05$).
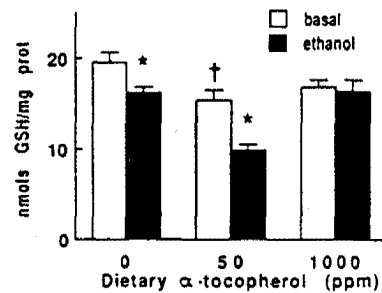

Fig. 2. Influence of ethanol consumption upon hepatic glutathione content in rats receiving varying amounts of dietary α-tocopherol. Each value represents the mean ± SE derived from six individual rats. *, differs from the corresponding group not consuming ethanol; †, differs from the corresponding group not receiving α-tocopherol ($p > 0.05$).
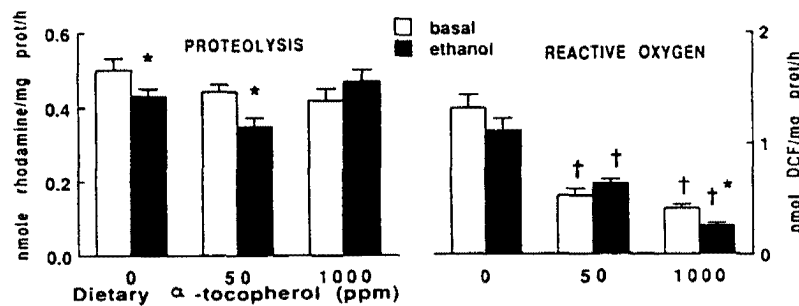

Fig. 3. Influence of ethanol consumption upon (left) proteolytic activity and (right) rate of generation of reactive oxygen species, in the liver of rats receiving varying amounts of dietary α-tocopherol. Each value represents the mean ± SE derived from six individual rats. *, differs from the corresponding group not consuming ethanol; †, differs from the corresponding group not receiving α-tocopherol ($p > 0.05$).
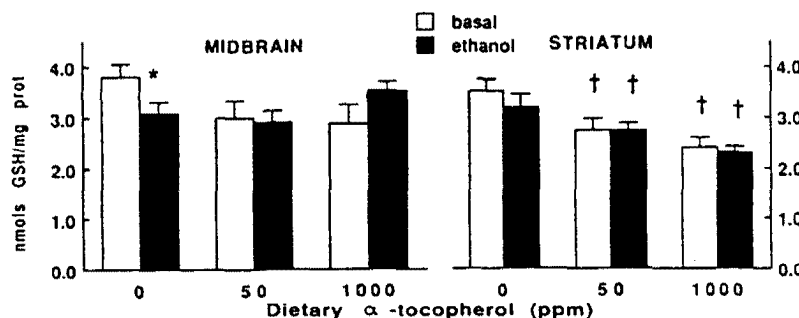

Fig. 4. Influence of ethanol consumption upon glutathione content of (left) midbrain and (right) striatum, from rats receiving varying amounts of dietary α-tocopherol. Each value represents the mean ± SE derived from six individual rats. *, differs from the corresponding group not consuming ethanol; †, differs from the corresponding group not receiving α-tocopherol ($p > 0.05$).

In animals not exposed to ethanol, hepatic glutathione levels were lowest in the group receiving moderate α-tocopherol (50 ppm). The presence of ethanol in the drinking water of 0 and 50 ppm groups reduced glutathione levels relative to the corresponding groups without ethanol in the drinking water. However, ethanol consumption had no effect on glutathione levels in rats receiving highest dietary α-tocopherol (Fig. 2).

Rates of proteolysis in the cytosolic fraction from the liver, were unaltered by the amount of α-tocopherol in the diet. However the concurrent presence of ethanol in the drinking water depressed proteolytic activity in the rats receiving no or moderate (50 ppm) dietary α-tocopherol but not when supplementation with this vitamin was maximal (Fig. 3). Thus high doses of this vitamin prevented the ethanol-induced reduction of hepatic protein degradation.

Rates of generation of ROS in the hepatic P2 were depressed by increasing dietary α-tocopherol in a dose-related manner. This reduction was only influenced by the presence of ethanol in the drinking water at the highest level of the vitamin where the ethanol further reduced ROS production. (Fig. 3).

Within neural tissue, absence of α-tocopherol in the diet, led to an elevation of glutathione levels in the midbrain and a similar nonsignificant trend was apparent in the striatum. (Fig. 4). In the case of the midbrain, but not the striatum, this elevation was abolished in the ethanol-consuming, α-tocopherol deficient group of rats. Glutathione content of the cerebral cortex and cerebellum was unaffected by either α-tocopherol or ethanol treatments (data not shown).

4. Discussion

All dosing in this study was by way of the diet. The degree of ethanol consumption in this experiment was moderate and normal weight gain was not affected. The extent of ethanol consumption is within levels found in human alcoholism, and would be paralleled by daily drinking of 290 g ethanol for a 70 kg individual This is equivalent to little more than a bottle of spirits each day. The amount of α-tocopherol in the diet was also relatively modest and, in the high dose group, resulted in an intake of about 88 mg α-tocopherol/kg body weight/day. This value is about six-fold greater than the daily ingestion of this vitamin by individuals utilizing it as a dietary supplement (up to 1 g daily by a 70 kg individual = 14 mg/kg).

Although the duration of exposure to the diets deficient in α-tocopherol, was only three weeks duration, signs of deficiency were evident in the liver since rates of ROS generation were elevated and glutathione levels were depressed relative to the group receiving moderate (50 ppm) α-tocopherol supplementation. A dose-response relation was evident in the case of DCF production relative to dietary α-tocopherol, but basal levels of glutathione were

elevated in the α-tocopherol deficient diet relative to the intermediate diet (Fig. 2). This may reflect the biphasic response of glutathione levels to pro-oxidant status (Adams et al., 1993). Parallel changes in cerebral regions of α-tocopherol deficient rats were generally not apparent, except that striatal and midbrain glutathione levels were elevated relative to both groups receiving α-tocopherol (Fig. 4). These regions may be especially vulnerable to oxidative stress (Liccione and Maines, 1988; Bondy and Guo, 1995), and this could account for their early responsiveness to α-tocopherol depletion.

Ethanol depressed hepatic glutathione levels in rats receiving either no, or minimal α-tocopherol in the diet. However ethanol did not lead to elevation in hepatic rates of ROS production any group tested. This may be due to the free radical-quenching properties of ethanol, which could obscure excess oxidative activity, detectable using the longer term index provided by glutathione levels.

The diet containing a high level of α-tocopherol prevented the ethanol-induced depression of liver proteolysis found in this study and in previous works (Donohue et al., 1989; Poso and Hirsimaki, 1991; Bondy and Guo, 1995). This finding was unexpected as there is no clear relation between elevated pro-oxidant status and inhibition of proteolytic activity. In general, excess generation of protein-damaging free radicals has been found to lead to induction of proteases (Dean, 1987; Davies, 1993). The protective effect of α-tocopherol may thus extend beyond the more immediate consequences of excess ROS formation.

Acknowledgements

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