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EFFECT OF CHRONIC ETHANOL FEEDING ON PLASMA AND LIVER α- AND γ-TOCOPHEROL LEVELS IN NORMAL AND VITAMIN E-DEFICIENT RATS

RELATIONSHIP TO LIPID PEROXIDATION

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Abstract—The effects of chronic ethanol intake on the levels of α-tocopherol and γ-tocopherol in serum and liver of both vitamin E-deficient and normal rats were studied. An intragastric feeding rat model was used. Both normal and vitamin E-deficient animals were fed a liquid diet and ethanol for 1 month. In pair-fed animals, dextrose was isocalorically replaced by ethanol. The blood ethanol level in the ethanol-fed animals was between 150 and 250 mg/dL. Liver peroxidation was determined by measuring thiobarbituric acid reactive substances (TBARS). Plasma alanine aminotransferase (ALT) was increased by 3-fold in vitamin E-deficient ethanol-fed rats compared with normal ethanol-fed rats. Plasma α- and γ-tocopherol were decreased in the normal ethanol-fed rats by 22.3 and 65%, respectively ($P < 0.01$). Liver α- and γ-tocopherol were also decreased by 51.7 and 76%, respectively ($P < 0.01$). Vitamin E-deficient animals had significantly lower mean plasma α-tocopherol (5670 vs 530 ng/mL, $P < 0.01$), and ethanol feeding did not decrease the levels any further. However, ethanol feeding decreased liver α- and γ-tocopherol by 58.5 and 56.5% ($P < 0.01$), respectively, beyond the already low levels observed in this group. There was an inverse correlation between liver TBARS and liver α-tocopherol ($r = -0.59$, $P < 0.05$) and γ-tocopherol ($r = -0.65$, $P < 0.02$). Also of significance is that ethanol feeding decreased the plasma and liver γ-tocopherol more than the α-tocopherol in both normal and vitamin E-deficient animals. In conclusion, ethanol feeding markedly decreased both α- and γ-tocopherol in livers of normal and vitamin E-deficient rats, but it only decreased plasma levels of tocopherols in normal rats. The higher ALT in vitamin E-deficient animals and the inverse correlation between TBARS and α- and γ-tocopherol suggest that enhanced lipid peroxidation is associated with greater severity of liver injury induced by ethanol in vitamin E-deficient rats.

Key words: ethanol; vitamin E; vitamin E-deficient rats; liver

Ethanol metabolism is associated with the generation of reactive oxygen species [1]. These oxygen free radicals are capable of initiating and/or enhancing peroxidation of polyunsaturated fatty acids [1]. Lipid peroxidation is a chain reaction that, once initiated, is self-perpetuating and can result in the oxidative deterioration of polyunsaturated fatty acids [2]. Although the exact mechanism(s) for ethanol-induced hepatotoxicity is not known, reactive oxygen species and/or lipid peroxidation may be responsible for ethanol-induced liver damage [3].

Vitamin E is a non-toxic potent antioxidant, and its role as an inhibitor, "chain blocker," of lipid peroxidation is well established [4]. Vitamin E is a general term that refers to the eight different forms: α-, β-, γ-, and δ-tocopherol with a chromanol ring and a saturated phytyl side chain, and four compounds (α-, β-, γ-, and δ-tocotrienol) with an unsaturated side chain. α-Tocopherol is the most potent of the different isoforms [5]. Vegetable oils in the Western diet provide substantial amounts of γ-tocopherol, which has one-tenth the biological activity of α-tocopherol.

Chronic ethanol feeding has been shown to alter vitamin E metabolism [6]. Serum vitamin E levels are reported to be reduced in alcoholics, although this may be due, in part, to malabsorption and low intake [7, 8]. Also, a decrease in the α-tocopherol content of liver occurs following chronic ethanol intake [9]. In addition, Meydani *et al.* [10] have shown a 50% reduction in the α-tocopherol/lipid ratio in the liver of ethanol-fed animals compared with that of pair-fed controls.

The present work was undertaken to investigate further the role of vitamin E in a model of chronic ethanol feeding. We used an intragastric feeding rat model to study plasma and liver α- and γ-tocopherol levels in both vitamin E-deficient and normal rats. Furthermore, since vitamin E and lipid peroxidation are closely interlinked, we evaluated the relationship between liver α- and γ-tocopherol content and lipid peroxidation.

MATERIALS AND METHODS

Animals and diet

The experimental animals were male Wistar rats

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weighing between 225 and 250 g. All animals were fed a liquid diet by continuous infusion through permanently implanted gastric cannulas, as described previously [11, 12]. The liquid diet was started at 12 mL/100 g body weight with a volume not exceeding 50 mL/day. The average caloric distribution of each nutrient was 25% of total calories as fat, 21% as protein, 12% as carbohydrate and 42% as ethanol or dextrose. Experimental and control groups (vitamin E sufficient and deficient) received the diet containing corn oil and dextrose (control groups) or ethanol. The ethanol-fed group received the same diet as the control except that ethanol was replaced isocalorically by dextrose. Vitamin E-deficient animals received a vitamin E-deficient diet containing vitamin E-stripped (Tecklad, Madison, WI) corn oil with either dextrose or ethanol. The amount of ethanol given was initially 8 g/kg/day and was increased up to 17 g/kg/day as tolerance developed. Blood alcohol levels were maintained at between 150 and 250 mg/dL. Animals were killed following 1 month of feeding. The liver was perfused with iron-free ice-cold saline, cut into small pieces, and frozen immediately in liquid nitrogen. A small piece of liver was also obtained for histologic analysis.

Biochemical analyses

To monitor the blood alcohol level, blood was collected from the tail vein at different periods of study, and ethanol concentration was measured using an alcohol dehydrogenase kit (Sigma Chemical Co., St. Louis, MO) [13]. Serum ALT* was measured using an automated method in routine use in our clinical laboratories.

Vitamin E determination

Plasma vitamin E. Plasma vitamin E was measured by HPLC using a previously described method [14]. Briefly, tocol was used as an internal standard in ethanol containing 0.1% BHT, which was added into 100 μL of a plasma aliquot and mixed vigorously. After deproteinization, tocopherols were extracted into hexane and reconstituted in 60 μL of methanol. α- and γ-Tocopherols were separated by a 5 μm C18 reverse phase column using 100% methanol for the mobile phase. Eluted peaks were detected using a Perkin–Elmer 650-15 fluorometer set at 292 nm excitation and 330 nm emission. Peaks were integrated by the Waters 860 system.

Liver vitamin E. One milliliter of ethanol containing 1.2% pyrogallol was added to 1 mL of tissue homogenate in phosphate buffer (100 mg/5 mL) and mixed. Following the addition of 60% KOH, the mixture was heated at 70° for 30 min. After cooling, 1 mL of distilled water, 1 mL of hexane containing tocol internal standard and 2 mL of hexane were added and shaken for 2 min. Following separation by centrifugation, 3 mL of the hexane layer was taken and dried under N_2, dissolved in 60 μL methanol, and injected into the HPLC system, as described above.

* Abbreviations: ALT, alanine aminotransferase; TBARS, thiobarbituric acid reactive substances; and BHT, butylated hydroxytoluene.

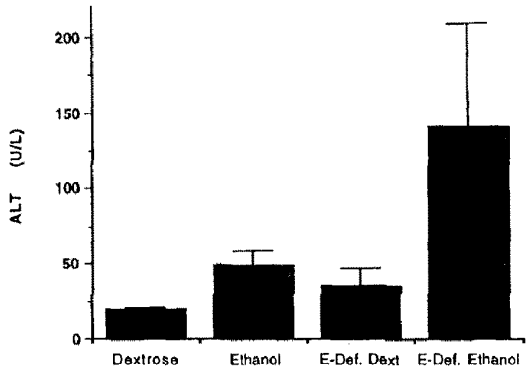

Fig. 1. Plasma alanine aminotransferase activity (U/L) in various experimental groups. Serum ALT in ethanol-fed rats (N = 3) was significantly different from that of dextrose-fed controls (N = 3) ($P < 0.001$). Vitamin E-deficient rats fed ethanol (N = 4) had significantly different ALT activity than the vitamin E-sufficient (normal) ethanol-fed group (N = 3) ($P < 0.01$) and the vitamin E-deficient dextrose group (N = 4) ($P < 0.01$). Values are means ± SD.

Tissue TBARS determination

Liver TBARS were measured as described previously [15]. Briefly, 0.2 mL of SDS (8.1%), 1.5 mL of acetic acid (20%) and 1.5 mL of 0.8% thiobarbituric acid was added to 200 μL of the homogenate. The final volume was brought to 4 mL with dH_2O. Tubes were vortexed and placed in boiling water for 1 hr, following which the reaction was stopped by immersion in a cold water bath. dH_2O (1.0 mL) and 5.0 mL of 15:1 (v/v) butanol:pyridine were added, and the tubes were vortexed and centrifuged for 10 min. The upper phase was removed and absorbance at 532 nm was determined. BHT was added to prevent TBARS formation during the assay.

Histologic analysis

When the rats were killed, a small sample of liver was obtained and formalin-fixed. Hematoxylin and eosin stain were used for light microscopy. The examination was carried out by a pathologist, who had no prior knowledge of the treatment groups. The liver pathology was scored as follows [11]: steatosis (the percentage of liver cells containing fat): 1 + = 0–25% of cells containing fat, 2 + = 26–50%, 3 + = 51–75%, 4 + = > 75%; inflammation and necrosis—one focus/lobule = 1 + , two or more foci/lobule = 2 + . The total pathology score was calculated by adding the scores from each of the parameters.

Statistical analysis

Results are presented as means ± SD. Comparison between groups was performed using Student's *t*-test. Pearson's correlation coefficient, *r*, was used to evaluate associations between the parameters.

RESULTS

The severity of liver damage following ethanol administration in the various groups is shown in Fig. 1. The ALT levels in ethanol-fed rats (50 ± 9 U/L)

Table 1. Changes in α- and γ-tocopherol levels in liver and plasma of vitamin E-sufficient and vitamin E-deficient animals fed dextrose or ethanol

	α-Tocopherol		γ-Tocopherol	
	Plasma (ng/mL)	Liver (ng/g)	Plasma (ng/mL)	Liver (ng/g)
Vitamin E sufficient				
Dextrose (N = 3)	5670 ± 170	5600 ± 110	240 (mean)	170 ± 20
Ethanol (N = 3)	4400 ± 70*	2700 ± 810†	84 ± 9	40 ± 10*
Vitamin E deficient				
Dextrose (N = 4)	530 ± 47	65 ± 11	62 ± 8	4.6 ± 0.8
Ethanol (N = 4)	640 ± 41	27 ± 5*	67 ± 10	2 ± 0.5*

Values are means ± SD.
* $P < 0.001$ compared with dextrose-fed rats in the same dietary vitamin E groups.
† $P < 0.01$ compared with dextrose-fed rats in the same dietary vitamin E groups.

were significantly higher ($P < 0.001$) than ALT levels in dextrose-fed controls (19 ± 2). Vitamin E-deficient animals fed ethanol had significantly higher ALT activity (141 ± 68) than the vitamin E-sufficient ethanol-fed rats (50 ± 9) or the vitamin E-deficient dextrose group (35 ± 12) ($P < 0.01$). The total pathology score in the vitamin E-deficient ethanol-fed group was higher (4.6 ± 0.9) but not significantly different from the vitamin E-sufficient ethanol-fed group (3.8 ± 0.6). However, the number of necrotic and inflammation foci were much greater in the former group, as reflected in the higher ALT levels.

The levels of α- and γ-tocopherol in plasma and liver of the various experimental groups are shown in Table 1. Compared with pair-fed dextrose controls, ethanol-fed rats showed a 22% decrease in plasma and a 52% decrease in liver α-tocopherol. The respective decreases in γ-tocopherol in the same group of animals were much greater (65% for plasma, 76% for liver). To compare the effect of ethanol on liver versus plasma tocopherol levels, we evaluated the liver:plasma α- and γ-tocopherol ratios in different experimental groups. The greater decrease in liver tocopherol levels compared with those in plasma is shown in Figs. 2 and 3. The changes in the mean liver:plasma ratio for both α- and γ-tocopherol suggest that the liver tocopherols are more susceptible to the effect of ethanol than the plasma tocopherols. The mean liver:plasma α-tocopherol ratio decreased from 0.99 (±0.12) in the dextrose-fed vitamin E-sufficient group to 0.61 (±0.14) in the ethanol group. In the vitamin E-deficient group, the ratio decreased from 0.12 ± 0.02 (dextrose-fed) to 0.04 ± 0.01 (ethanol-fed). A similar change was seen in the liver:plasma γ-tocopherol ratio (from 0.71 to 0.47 in normal rats, and from 0.1 ± 0.02 to 0.03 ± 0.01 in the vitamin E-deficient group).

To determine which form of the tocopherols (α or γ) showed a greater decrease in response to ethanol, we compared the γ:α tocopherol ratios in both plasma and liver (Figs. 4 and 5). In plasma, the mean γ:α ratio decreased by 50% in vitamin E-sufficient rats (0.042 to 0.019). There was no significant change in this ratio in vitamin E-deficient rats (0.12 ± 0.003 to 0.11 ± 0.014). The liver γ:α ratio was also decreased from 0.030 to 0.014 in

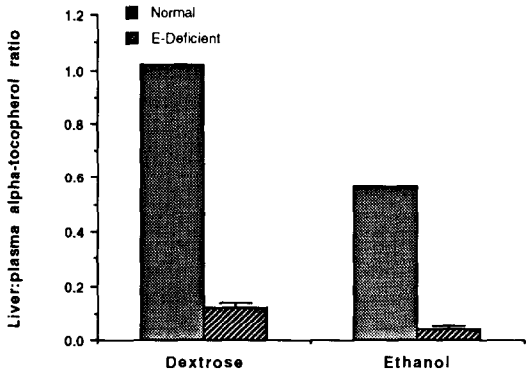

Fig. 2. Changes in the liver:plasma α-tocopherol ratio in response to ethanol in both normal and vitamin E-deficient rats. In both normal rats (N = 3) and the vitamin E-deficient group (N = 4), the liver:plasma ratio decreased significantly ($P < 0.001$). Values are means ± SD. The error bars for normal animals are too small to be seen.

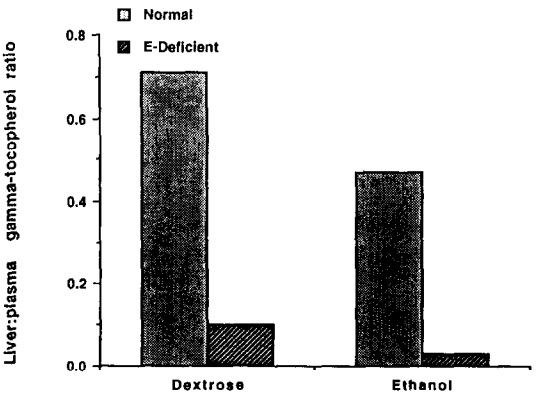

Fig. 3. Changes in the liver:plasma γ-tocopherol ratio in response to ethanol in both normal and vitamin E-deficient rats. In normal rats (N = 3) and in the vitamin E-deficient group (N = 4), the ratio decreased significantly ($P < 0.01$). Values are means ± SD. The error bars are too small to be seen.

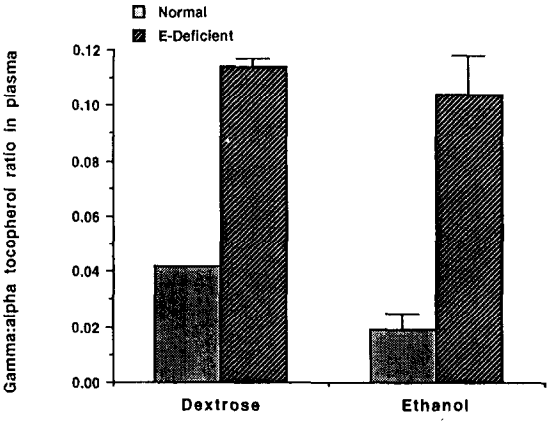

Fig. 4. Changes in the plasma γ:α tocopherol ratio in response to ethanol feeding in both normal and vitamin E-deficient rats. In normal rats, the mean ratio decreased significantly ($P < 0.01$). Values are means ± SD; N = 3 for normal rats and N = 4 for vitamin E-deficient rats. The error bar for the dextrose-fed normal group is too small to be seen.

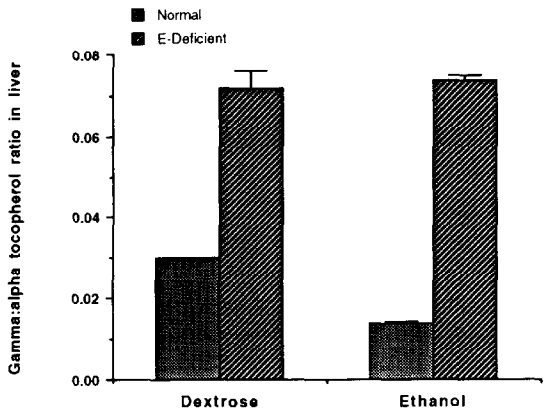

Fig. 5. Changes in the liver γ:α tocopherol ratio in response to ethanol feeding in both normal and vitamin E-deficient rats. In normal rats, the mean ratio decreased significantly ($P < 0.01$). Values are means ± SD; N = 3 for normal rats and N = 4 for vitamin E-deficient rats. Error bars for normal animals are too small to be seen.

normal rats, but again the ratio in vitamin E-deficient rats did not change significantly. These results in vitamin E-sufficient animals show that both α- and γ-tocopherol levels decreased in response to ethanol. However, the decrease in liver levels was greater than that seen in plasma. Furthermore, γ-tocopherol decreased to a greater extent than α-tocopherol, especially in the liver.

Vitamin E-deficient dextrose-fed animals had an approximately 10-fold lower level of plasma α-tocopherol and a 4-fold lower level of plasma γ-tocopherol than did vitamin E-sufficient controls. The decline in liver α- and γ-tocopherol content was greater than that seen in plasma. For example, the α-tocopherol level in the liver of vitamin E-deficient animals was about 1% of that in the vitamin E-sufficient group; γ-tocopherol was about 3% (Table 1), whereas the plasma concentration of α-tocopherol in the vitamin E-deficient animals was only 10 times lower than that of the vitamin E-sufficient group.

Ethanol feeding did not have a significant effect on the plasma concentration of either α- or γ-tocopherol in vitamin E-deficient animals. However, a significant decrease was seen with both α- and γ-tocopherol levels in the liver (Table 1). This decrease in liver but not in plasma levels is reflected in the liver:plasma ratios of α- and γ-tocopherol (Figs. 2 and 3). A 65% decrease of this ratio was observed for α-tocopherol (0.12 ± 0.02 to 0.04 ± 0.01, $P < 0.001$), and for γ-tocopherol (0.1 ± 0.02 to 0.03 ± 0.01, $P < 0.01$).

The difference in the changes in the γ:α ratios in plasma and liver in both vitamin E-deficient and -sufficient rats is shown in Figs. 4 and 5. In contrast to the ethanol-induced decrease in the γ:α ratio seen in vitamin E-sufficient rats, the ratio remained unchanged in vitamin E-deficient rats. This absence of change in the liver γ:α ratio in vitamin E-deficient rats is explained by the fact that both α- and γ-tocopherol levels in liver decreased to the same degree (56–58%). This is in contrast to the greater drop in liver γ-tocopherol (76%) than in α-tocopherol (50%) in vitamin E-sufficient animals, which led to a decrease in the γ:α ratio.

The relationship between liver vitamin E and lipid peroxidation is shown in Fig. 6. Figure 6A shows a significant inverse correlation between liver α-tocopherol and liver TBARS ($r = -0.59$, $P < 0.05$). A similar significant inverse correlation was seen between liver TBARS and γ-tocopherol (Fig. 6B, $r = -0.65$, $P < 0.02$). These results suggest that enhanced lipid peroxidation is associated with low levels of liver vitamin E, which, in turn, are associated with the enhanced severity of the liver injury (Fig. 1). The present study not only confirms previous observations showing that ethanol feeding results in decreased hepatic and plasma tocopherol levels, but in addition shows that the decrease in γ-tocopherol is more pronounced than the decrease in α-tocopherol and that both tocopherols correlated inversely with the degree of lipid peroxidation. In addition, we also demonstrated that in vitamin E-deficient animals the decrease in α-tocopherol was more pronounced than that of γ-tocopherol, suggesting that the latter is relatively better preserved in the vitamin E-deficient groups.

DISCUSSION

Vitamin E is a potent fat-soluble antioxidant that protects biological membranes against the damaging effects of reactive oxygen species [5]. The mechanism(s) of reduction in hepatic and plasma α-tocopherol after chronic ethanol feeding is unknown. Possible mechanisms include decreased intestinal absorption, impaired lipoprotein uptake by hepatocytes, increased mobilization of tocopherol from the liver and increased conversion of α-tocopherol to α-tocopherol quinone [6]. The last possibility is

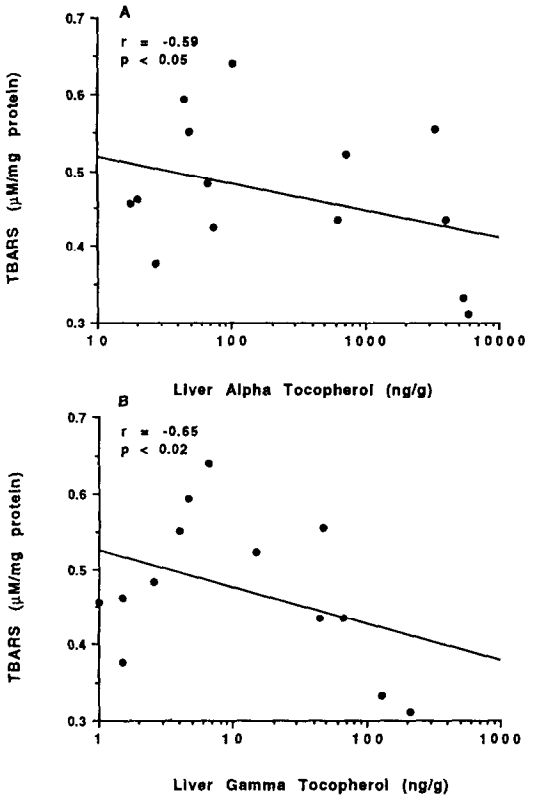

Fig. 6. (A) Significant inverse correlation between liver α-tocopherol and liver TBARS ($r = -0.59$, $P < 0.05$). (B) Significant inverse correlation between liver γ-tocopherol and liver TBARS ($r = -0.65$, $P < 0.02$). Each point in the two panels represents an individual animal.

suggested by previous observations in the intragastric feeding model where increased levels of lipid peroxidation in the liver have been observed [16]. Other studies have also shown increased lipid peroxidation in other models of chronic ethanol feeding [17]. *In vitro* studies have shown that exposure of liver hepatocytes to ethanol results in a decrease in the cellular content of α-tocopherol and an increase in the α-tocopherol turnover rate [18]. The present work demonstrates that vitamin E-deficient animals are more susceptible to ethanol-induced liver damage, as shown by the high serum ALT values in those animals compared with values of the normal rats. We also showed an inverse correlation between liver TBARS and α- and γ-tocopherol. Therefore, it seems logical to assume that enhanced lipid peroxidation is associated with greater severity of liver injury in vitamin E-deficient rats.

Several studies have shown that chronic ethanol feeding alters α- and γ-tocopherol in the liver [6, 8–10]. Meydani *et al.* [10] reported a 44% reduction in liver α-tocopherol/mg of total lipid. Our studies, which are consistent with those of Meydani *et al.* [10], showed a 51.7% reduction in liver α-tocopherol and a 22.3% reduction in plasma α-tocopherol following chronic ethanol feeding (Table 1). In addition, in the vitamin E-sufficient group, γ-tocopherol was reduced to a greater extent in both the liver (76%) and the plasma (65%). Note that reduction in both α- and γ-tocopherol was greater in liver than in plasma. In vitamin E-deficient animals, the decrease in both α- and γ-tocopherol was seen only in liver (Table 1). The exact significance of these findings related to γ-tocopherol is unclear, since γ-tocopherol has only 10% of the biologic antioxidant effect of α-tocopherol and is not likely to provide significant antioxidant effects.

The main conclusions of our study are: (1) vitamin E deficiency is associated with enhanced lipid peroxidation and greater severity of liver injury in response to ethanol feeding; (2) in vitamin E-sufficient animals, the decrease in liver tocopherol (both α and γ) levels is greater than the concomitant decrease in plasma levels. This greater decrease in liver tocopherol levels is probably secondary to enhanced lipid peroxidation in the liver, since alcohol is metabolized in the liver and enhances lipid peroxidation. The oxidation of vitamin E by lipid peroxides [6] is one possible reason for the lower liver levels; (3) in vitamin E-sufficient rats, feeding ethanol decreases γ-tocopherol (in both liver and plasma) more than α-tocopherol; and (4) in vitamin E-deficient rats, ethanol has no effect on plasma α- or γ-tocopherol concentration but significantly decreases liver α- and γ-tocopherol antioxidant defenses, suggesting that in both vitamin E-sufficient and -deficient rats the liver tocopherols are utilized to defend against oxidative stress induced by ethanol feeding.

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