REGULATION OF MANGANESE SUPEROXYDE DISMUTASE (MnSOD) IN CHRONIC EXPERIMENTAL ALCOHOLISM: EFFECTS OF VITAMIN E-SUPPLEMENTED AND -DEFICIENT DIETS

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Abstract — In order to investigate the pathogenic mechanism responsible for liver injury associated with chronic alcoholism, we studied the effects of different dietary vitamin E levels in chronically ethanol (EtOH)-fed rats on the activity and mRNA regulation of the manganese superoxide dismutase (MnSOD) enzyme. Evidence is accumulating that intermediates of oxygen reduction may in fact be associated with the development of alcoholic liver disease. Since low vitamin E liver content seems to potentiate EtOH-linked oxidative stress, we studied the effect of EtOH treatment in livers from rats fed a diet deficient or supplemented with vitamin E. Chronic EtOH feeding enhanced hepatic consumption of vitamin E in both groups of EtOH-treated animals, irrespectively of the vitamin E level of the basal diet and the effect was observed in both the microsomal and mitochondrial fractions. Both EtOH-fed groups exhibited increased MnSOD gene expression, while the enzyme activity was enhanced only in the vitamin E-deprived group of EtOH-treated animals. The significant increase in manganese liver content found only in this last group could explain the rise of enzyme activity. In fact, in the absence of a parallel increase of the prosthetic ion manganese, MnSOD mRNA induction was not accompanied by a higher enzymatic activity. These findings support the role of oxidative alteration in the EtOH-induced chronic hepatotoxicity in which MnSOD response might represent a primary defence mechanism against the damaging effect of oxygen radical species.

INTRODUCTION

Although many theories have been advanced, the pathogenic mechanism underlying liver injury associated with chronic alcoholism remains unsettled. Since the pioneering report of Di Luzio (Di Luzio, 1973), much evidence has been provided that free radicals are usually implicated in this process (Koch et al., 1991; Nordmann et al., 1992; Lieber, 1997). Besides electron-spin resonance (ESR) detection (Albano et al., 1988; Reinke et al., 1997) in liver microsomes isolated from ethanol (EtOH)-fed rats of EtOH- and lipid-derived free radicals, reactive oxygen species (ROS) have been reported to accumulate in various subcellular compartments of hepatocytes after EtOH consumption (Nordmann et al., 1992). Such a condition of oxidative-stress is frequently associated with enhanced lipid-peroxidation, as established by the use of non-invasive techniques (Sies et al., 1979; Boveris et al., 1983) and with protein (Remmer et al., 1989; Rouach et al., 1997) and DNA (Rajasinghe et al., 1990; Kurose et al., 1997) damage. Data are also available in the literature on the level of non-enzymatic antioxidants in the liver of chronic EtOH-fed rats (Nordmann et al., 1992). A previous report from our group (Koch et al., 1994) showed that rat liver after chronic EtOH feeding exhibited an increased manganese superoxide dismutase (MnSOD) activity with an upregulation of the enzyme at the mRNA level. The same study showed that chronic intake of EtOH also led to a significant decrease in the content of vitamin E in both the liver mitochondrial and microsomal fractions, suggesting the occurrence of enhanced lipid peroxidation.

According to some reports (Kawase et al., 1989; Sadrzadeh et al., 1994), low vitamin E liver content seems to potentiate the EtOH-linked oxidative stress; thus, it may be possible that different dietary vitamin E levels in chronically EtOH-fed rats may also modify the expression of the MnSOD mRNA. In order to evaluate this possibility, the present study explored the activity and RNA regulation of MnSOD in chronically EtOH-treated rats fed either a vitamin E-deficient or -supplemented diet.

MATERIALS AND METHODS

Animals and treatment

A total of 14 male Wistar rats (232–305 g initial body weight) was divided into two groups. One group (AD) was fed for 15 days a semi-synthetic basal diet deficient in vitamin E ad libitum (Table 1). The other group (AS) was fed the same diet supplemented with vitamin E (Table 1).

<table>
<thead>
<tr>
<th>Components</th>
<th>Vitamin E deficient (g/kg)</th>
<th>Vitamin E supplemented (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg albumin</td>
<td>420</td>
<td>420</td>
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<tr>
<td>Corn starch</td>
<td>86</td>
<td>86</td>
</tr>
<tr>
<td>Sucrose</td>
<td>240</td>
<td>238.5</td>
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<tr>
<td>Cellulose</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Coconut oil</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>Salt mixture</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>Oligoelements</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Vitamin mixture</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>—</td>
<td>1.5</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
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basal diet for a similar period but supplemented with vitamin E. Both groups were offered a mixture of 25% EtOH–20% sucrose as the only source of drinking fluid during the entire period of treatment (15 days). Another two control groups (seven animals each, CD: control — vitamin E deficient and CS: control — vitamin E supplemented) were offered water and their solid diets were based on the final regimens (basal diet plus EtOH–sucrose solution) consumed by the alcohol groups, but EtOH was replaced isocalorically by a mixture of 50% sucrose–50% corn starch. These control animals were pair-fed isocalorically with those of the alcohol groups. The caloric percentages of ingredients of the final regimen (basal diet plus EtOH–sucrose solution) were as follows: Group AD: alcohol 38%, carbohydrates 37%, fat 7%, protein 18%; Group AS: alcohol 41%, carbohydrates 35%, fat 7%, protein 17%. The composition of the diet of the control groups was the same, except that the alcohol-derived calories were replaced by carbohydrates. Animals in the alcohol groups were kept without EtOH for 18 h before death, but were allowed free access to drinking water. Animals from all groups were starved of food overnight.

**RNA isolation and quantitative analysis**

RNA was isolated essentially according to Chirgwin et al. (1979). Total RNA was size-fractionated by formaldehyde–agarose (1.5%) gel electrophoresis, and blotted to nylon membranes. Blots were pre-hybridized at 42°C for 5 h and hybridized overnight to rat MnSOD cDNA probe radiolabelled by the Multiprime DNA labelling system (Amer sham, UK). Autoradiograms were obtained at −70°C using Kodak XAR film. The intensity of autoradiograms was scanned with a transmission densitometer.

**Enzyme assay**

Copper–zinc superoxide dismutase (CuZnSOD) and MnSOD activities were determined in 48 h-dialysed supernatants (105 000 g) of homogenates of liver specimens, by inhibition of haematoxylin to haematein autoxidation monitored at 560 nm, at pH 7.5 and 25°C (Martin et al., 1987). MnSOD was measured in the presence of 3.0 mM cyanide.

**Vitamin E evaluation**

Vitamin E was extracted as indicated by Handelman et al. (1988). The samples were dissolved in methanol and 20 µl aliquots were analysed by reverse phase HPLC with fluorescence detection using a Perkin–Elmer 650 LC fluorescence detector, with excitation at 295 nm and emission at 340 nm. Vitamin E, as well as the internal standard, tocol, was eluted with 100% methanol on an Alltech C18 3-µm column, as described previously (Palozza et al., 1992).

**Metal determination**

Metal concentrations were determined by atomic absorption spectrometry using a Perkin–Elmer 272 spectrophotometer. Thin slices of liver sample were dried at 100°C, digested with nitric acid and then analysed for metal content.

**Statistical analysis**

All data are presented as the means ± SEM. We estimated differences among the different treatments by one-way analysis of variance (ANOVA), using Minitab software (Minitab, State College, PA, USA). When a significant effect was found, post-hoc comparisons of means were made using Fisher’s test. Differences were considered significant at \( P < 0.05 \).

**RESULTS**

Figure 1 shows the effect of EtOH treatment on the vitamin E content of liver microsomal (A) and mitochondrial (B) membranes from rats fed a diet deficient or supplemented with vitamin E. EtOH treatment reduced vitamin E content of rat liver drastically; this effect was observed in both microsomal and mitochondrial membranes.

MnSOD activities in all groups are indicated in Table 2. Activity was higher only in the vitamin E-deprived group of EtOH-treated animals, compared to the respective control.

**Fig 1.** Effect of a 15-day ethanol (EtOH) treatment on vitamin E content of liver microsomal (A) and mitochondrial (B) membranes from rats fed a diet deficient or supplemented with vitamin E. Values are means ± SEM of three experiments. Values not sharing the same letter are significantly different, \( P < 0.05 \).

**Abbreviations used:** CS, control vitamin E supplemented; AS, vitamin E supplemented + EtOH; CD, control vitamin E deficient; AD, vitamin E deficient + EtOH.
Table 2. Superoxide dismutase activities in livers of vitamin E-deficient and -supplemented rats after ethanol (EtOH) treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MnSOD (μg/mg wet. wt)</th>
<th>CuZnSOD (μg/mg wet. wt)</th>
</tr>
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<tbody>
<tr>
<td>CD</td>
<td>198.0 ± 0.7 (4)</td>
<td>559.5 ± 34 (4)</td>
</tr>
<tr>
<td>AD</td>
<td>229.0 ± 24 (4)*</td>
<td>465.8 ± 65 (4)</td>
</tr>
<tr>
<td>CS</td>
<td>224.3 ± 14.7 (4)</td>
<td>558.8 ± 68 (4)</td>
</tr>
<tr>
<td>AS</td>
<td>218.0 ± 18 (4)</td>
<td>428.8 ± 51 (4)</td>
</tr>
</tbody>
</table>

Abbreviations used: CD, control vitamin E-deficient; AD, vitamin E-deficient + EtOH; CS, control vitamin E-supplemented; AS, vitamin E-supplemented + EtOH.

*P < 0.05 vs vitamin E-deficient group. Values are means ± SEM (of the numbers of observations on parentheses).

Fig 2. Levels of MnSOD mRNA in liver of vitamin E-deficient and -supplemented ethanol (EtOH)-treated rats.

Values of the treated animals are means ± SEM of three experiments. Uniformity of lane to lane loading was confirmed by inspection of the ethidium bromide-stained gel. MnSOD mRNA levels correspond to the sum of the intensities of the two major hybridizing bands (1.08 and 0.85 kb), normalized to 100% from control liver. *P < 0.05 vs vitamin E-deficient group.

**P < 0.05 vs vitamin E-supplemented group.

Abbreviations used: CD, control vitamin E-deficient; AD, vitamin E deficient + EtOH; CS, control vitamin E supplemented; AS, vitamin E supplemented + EtOH.

DISCUSSION

The present report clearly confirms that chronic EtOH intake enhances hepatic consumption of vitamin E, probably caused by an increment in the formation of ROS induced directly or indirectly by EtOH itself. This increase in hepatic vitamin E consumption was observed in both groups of EtOH-treated animals, irrespective of the vitamin E levels of the basal diets. These data are in agreement with those reported in the literature showing a reduction in hepatic vitamin E content after chronic EtOH administration (Bjorneboe et al., 1987; Kawase et al., 1989; Koch et al., 1994; Sadrazadeh et al., 1994; Rouach et al., 1997). In a previous study, we reported that an EtOH diet was able to reduce significantly the hepatic vitamin E content in rats fed a normal control diet, containing the usual recommended allowances of vitamin E of 0.6 mg/kg body wt/day (National Research Council, 1995). In the present study, this effect was observed in both the microsomal and mitochondrial fractions. In contrast, no differences in the content of mitochondrial or microsomal vitamin E by EtOH were reported by Kawase et al. (1989), although these authors found a marked reduction in levels of antioxidant in whole liver. These controversial results may be explained by the model of chronic alcoholism employed. Liquid EtOH diets with high lipid contents which substantially differ from those used in the present study were used. The mechanism by which EtOH decreases vitamin E is still under investigation. It is possible that EtOH causes an over-production of ROS either directly or indirectly.

In this study, it remains still unclear why vitamin E is altered differently by the same dose of EtOH depending on dietary administration of the vitamin E itself. This might be explained in different ways. It is possible that different pools of vitamin E near the surfaces or in the depths of membranes...
might be present in liver as a consequence of a different vitamin E intake, with consequently different susceptibility to free radical species (Palozza et al., 1992). Alternatively, free radical species may be formed during oxidative stress induced by EtOH, against which even high levels of vitamin E are not effective.

MnSOD is one of the enzymatic antioxidants whose action may be referred to primarily as the ability to change the intramitochondrial levels of ROS. In a previous report (Koch et al., 1994), we have shown that chronic EtOH feeding caused an upregulation of the enzyme at the mRNA level, with a good correlation between the transcript and the enzyme activity. The results reported here extend these observations and clearly demonstrate that the increase in the MnSOD mRNA does not correlate with microsomal or mitochondrial vitamin E levels. In this regard, rats fed with EtOH plus vitamin E have still higher hepatic levels of vitamin E than non-EtOH rats fed stock diets. Although both EtOH-fed groups exhibit increased MnSOD gene expression, a parallel increase in the enzyme activity is observed only in the EtOH-treated vitamin E-deficient animal group, which is the only one characterized by a significant increase in the manganese ion. It is possible, therefore, that lower levels of Mn, such as those found in the EtOH-treated vitamin E-supplemented group, are not sufficient to activate post-translationally higher amounts of the apoenzyme accumulated following the increased mRNA. Nanji et al. (1995) showed, in the gastric continuous EtOH infusion model, that rats treated for 1 month did not exhibit increases in the hepatic levels of MnSOD mRNA nor reduced levels when rats were fed totally liquid EtOH diets higher in unsaturated lipids, such as corn oil or fish oil. This apparent discrepancy may be explained by marked differences in the chronic EtOH models used by Nanji et al. (1995) and the one reported here.

Unlike the corresponding cytosolic enzyme CuZnSOD, which is constitutively expressed, MnSOD is easily inducible by different agents, such as cytokines, auto-oxidizable drugs and ionizing radiation. In this regard, it has been shown that chronic EtOH could enhance tumour necrosis factor (TNF) expression in rats (Nanji, et al., 1994; Perera, et al., 1995) playing an important role in the pathogenesis of both experimental and clinical liver damage (McLain and Cohen, 1989; McLain et al., 1993). Adachi et al. (1994) have shown that Kupffer cells, the main source of TNF, are activated by chronic EtOH treatment and that their inactivation is diminished early in EtOH-linked liver injury. It is noteworthy that a study performed on MnSOD in humans (Togashi et al., 1990) showed a clear increase in this enzyme activity in chronic alcoholics with alcoholic hepatitis or cirrhosis, compared with non-alcoholics with or without overt liver diseases.

We have proposed previously that the increase of MnSOD could be a protective mechanism built up by the genetic machinery to partially overcome EtOH-induced oxidative stress. However, in the case of chronic alcoholism, the increased activity of MnSOD may aggravate the antioxidant–pro-oxidant status of hepatic mitochondria, as suggested by Fernandez-Checa et al. (1997). In fact, mitochondrial hydrogen peroxide concentration may substantially increase owing to a depletion of reduced glutathione (GSH) with the subsequent lower activity of the hydrogen peroxide-metabolizing enzyme glutathione peroxidase (GPX). The depletion of GSH results from a primary defect of hepatic mitochondria from EtOH-fed rats to transport GSH from cytosol into the mitochondrial matrix (Fernandez-Checa et al., 1987; Hirano et al., 1992). In such a condition, an increase of MnSOD may aggravate the high steady-state concentration of hydrogen peroxide. Accumulation of hydrogen peroxide would then participate in the chemistry catalysed by transition metals that would give rise to generation of potent free radicals, such as OH·, and hence mitochondrial damage.

In conclusion, in animals fed chronically with EtOH, vitamin E supplementation is unable to prevent the EtOH damage and the consequent increased MnSOD expression. We believe that EtOH consumption may induce MnSOD by ROS generation against which vitamin E, at least in part, is not protective. However, in the absence of adequate activities of GPX, the induction of MnSOD may aggravate oxidatively mediated EtOH-induced mitochondrial injury.

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