Hepatic Mitochondrial Prooxidant and Antioxidant Status in Ethanol-Induced Liver Injury in Rats

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In this study, prooxidant and antioxidant status in liver homogenates and their mitochondrial fractions were investigated in both chronic and chronic plus acute ethanol-treated rats. Increases in serum transaminase activities, as well as increases in total lipid, triglyceride, malondialdehyde (MDA) and diene conjugate (DC) levels and decreases in glutathione (GSH), vitamin E and vitamin C levels, have been observed in liver homogenates following chronic ethanol treatment (20% ethanol, v/v as drinking water for 3 months), but CuZn-superoxide dismutase (CuZnSOD), glutathione peroxidase (GSH-Px) and glutathione transferase (GST) activities remained unchanged in postmitochondrial fractions. When an acute dose of ethanol (5 g/kg, i.p.) was given rats which had received ethanol chronically, serum transaminase activities and hepatic lipid and MDA and DC levels increased further, but GSH levels and antioxidant enzymes decreased more compared to the chronic ethanol-treated rats. There were no significant differences in the levels of MDA, DC and protein carbonyl and the activities of GSH-Px and GST in the hepatic mitochondrial fraction of rats following both chronic and chronic plus acute treatments. Mn-superoxide dismutase (MnSOD) activities increased in both groups, but mitochondrial GSH levels decreased only after chronic plus acute treatment. Therefore, we suggest that the increase in MnSOD activity may play an important role in the regulation of mitochondrial susceptibility against ethanol-induced oxidative stress.

Key words ethanol; liver; mitochondria; oxidative stress; antioxidants; rat

Hepatic mitochondrial fraction is a target organelle of ethanol intoxication. Therefore, mitochondrial oxidative stress has been accepted to play an important role in the pathogenesis and progression of alcoholic liver injury. Ethanol administration caused increases in oxygen consumption and the generation of reactive oxygen species (ROS) in hepatic mitochondria. ROS are also generated in mitochondria through acetaldehyde oxidation. ROS may cause damage to target proteins, lipids, and DNA of this subcellular organelle in the absence of adequate protection.

The mitochondrial antioxidant system, especially glutathione (GSH), plays an important role against oxidative stress in hepatic mitochondria. Since mitochondria are unable to synthesize GSH, mitochondrial GSH is obtained by specific uptake from the cytoplasmic pool. Mn-superoxide dismutase (MnSOD), and glutathione peroxidase (GSH-Px) are important enzymes functioning in the protection of mitochondria from oxidative stress, since mitochondria do not have catalase activity. Mitochondrial glutathione transferase (GST) may also play an important role against oxidative stress, like microsomal GSTs.

Although some studies have addressed the relationship between mitochondrial oxidative stress and ethanol toxicity, the results obtained from these studies are conflicting. Some investigators have reported increased mitochondrial lipid peroxide and protein carbonyl (PC) levels after chronic ethanol treatment, while some conflicting results have been presented. Unchanged decreased, and even elevated mitochondrial GSH levels have been reported in the literature after chronic ethanol treatment. Similar conflicting results on mitochondrial oxidative stress have also been presented in acute ethanol-treated animals. However, there are limited studies on mitochondrial antioxidant enzymes following ethanol treatment. Therefore, in this study, we wanted to investigate mitochondrial prooxidant and antioxidant status in chronic ethanol-treated rats. This investigation was also undertaken to determine the susceptibility of mitochondria to oxidative stress after the administration of an acute dose of ethanol to rats which received ethanol chronically.

MATERIALS AND METHODS

Rats and TreatmentMale Wistar rats (180—200 g) were used for all the experiments. Animals were obtained from the Experimental Medical Research Institute of Istanbul University. Rats were fed a standard diet and had free access to water. Ethanol was added to drinking water 20% (v/v) for 3 months (approximately 8.5 g ethanol/kg body weight/d). Control rats (n=8) were given tap water as a drinking fluid. At the end of this period, rats received ethanol chronically were divided into two groups. The first group was given an acute dose of ethanol (5 g/kg, i.p.) and the other group saline only. Rats 4 h after ethanol treatment were anesthetized with ether, and blood was drawn by cardiac puncture.

Experimental Procedure and Determinations Serum transaminase (ALT and AST) activities were determined using kits from Sigma. The livers were rapidly removed, washed in 0.9% NaCl and kept in ice. Liver portions were used for all the experiments. Animals were obtained from the Experimental Medical Research Institute of Istanbul University. Rats were fed a standard diet and had free access to water. Ethanol was added to drinking water 20% (v/v) for 3 months (approximately 8.5 g ethanol/kg body weight/d). Control rats (n=8) were given tap water as a drinking fluid. At the end of this period, rats received ethanol chronically were divided into two groups. The first group was given an acute dose of ethanol (5 g/kg, i.p.) and the other group saline only. Rats 4 h after ethanol treatment were anesthetized with ether, and blood was drawn by cardiac puncture.

Experimental Procedure and Determinations Serum transaminase (ALT and AST) activities were determined using kits from Sigma. The livers were rapidly removed, washed in 0.9% NaCl and kept in ice. Liver portions were homogenized in ice-cold 0.15 M KCl 10% (w/v). Mitochondria were obtained by sequential centrifugation. The homogenates were centrifuged at 600 × g for 10 min at below 4 °C, and the supernatants were centrifuged at 10000 × g for 20 min. The pellets were washed with ice-cold 0.15 M KCl, and after washing they were suspended in the same solution. Lipids were extracted by chloroform: methanol (2:1) and hepatic total lipid levels were measured according to the methods of Chiang et al. The degree of lipid peroxidation was assessed measuring malondialdehyde (MDA) levels using the thiobarbituric acid test (TBA) and diene conjugate levels in liver homogenates and mitochondrial fraction. PCs were measured according to the
method described by Reznick and Packer\textsuperscript{17} based on spectrophotometric detection of the reaction of 2,4-dinitrophenyl-hydrazine with protein carbonyl to form protein hydrazones. GSH levels in liver homogenates and mitochondrial fraction were measured according to the methods of Beutler et al.\textsuperscript{18} and Tietze,\textsuperscript{19} respectively. Liver vitamin E and vitamin C levels were determined in homogenates by the method of Desai\textsuperscript{20} and Omaye et al.\textsuperscript{21} respectively. SOD, GSH-Px, and GST activities were determined in the hepatic mitochondrial and postmitochondrial fractions. CuZnSOD and MnSOD activities were assayed by its ability to increase the effect of riboflavin-sensitized photooxidation of orthodaniisidine.\textsuperscript{22} MnSOD activity was measured in the presence of 4 mm cyanide. GSH-Px activities were measured using cumene hydroperoxide as a substrate.\textsuperscript{23} GST activities were determined using the spectrophotometric method using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate.\textsuperscript{24} Protein levels were determined by the method of Lowry et al.\textsuperscript{25} using bovine serum albumin as a standard.

**Statistical Analysis** Results are given as the mean \( \pm \) S.D. These data was analyzed by one-way analysis of variance (ANOVA) followed Tukey’s test. In all cases, a difference was considered significant when \( p<0.05 \).

**RESULTS AND DISCUSSION**

There is increasing evidence that ethanol-induced liver injury is associated with free radical injury and oxidative stress.\textsuperscript{26,27} Oxidative stress is characterized by increased lipid peroxidation and/or altered nonenzymatic and enzymatic antioxidant systems. However, conflicting results are reported in the literature concerning oxidative stress following chronic ethanol treatment in rats.\textsuperscript{28—30} These discrepancies may be due to the techniques used for assessing oxidative stress as well as varying conditions of ethanol administration. Several types of rat models are used for alcoholic liver disease studies, such as the Lieber–DeCarli liquid diet closer to human conditions than a liquid diet.\textsuperscript{32} Our protocol was used to cause significant liver damage in rats.\textsuperscript{7,28,33,34} Therefore, in the present study, rats were given water containing 20% (v/v) ethanol as drinking water for 3 months. We found that chronic ethanol treatment caused significant increases in plasma ALT and AST activities and hepatic total lipid, triglyceride, MDA, and PC levels, and significant decreases in GSH, vitamin E, and vitamin C levels, but hepatic CuZnSOD, GSH-Px and GST activities remained unchanged as compared with those in controls (Figs. 1, 2). Under these conditions, we have not observed any significant changes in MDA, DC or PC levels as well as GSH levels and GSH-Px or GST activities in hepatic mitochondrial fraction (Table 1). Only MnSOD activity was observed to increase mildly, but significantly. Some investigators have also reported that MnSOD activity increased following chronic ethanol treatment.\textsuperscript{10,13} Therefore, this increase may play an important role in the protection of mitochondria against ethanol-induced oxidative stress.

**Table 1.** Mitochondrial Malondialdehyde (MDA), Diene Conjugate (DC), Protein Carbonyl (PC) and Glutathione (GSH) Levels and Mn-Superoxide Dismutase (MnSOD), Glutathione Peroxidase (GSH-Px) and Glutathione Transferase (GST) Activities in the Liver Following Both Chronic and Chronic Plus Acute Ethanol Treatments in Rats (Mean \( \pm \) S.D.)

<table>
<thead>
<tr>
<th></th>
<th>Control (n=8)</th>
<th>Chronic ethanol (n=8)</th>
<th>Chronic plus acute ethanol (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (nmol/mg protein)</td>
<td>1.20( \pm )0.13</td>
<td>1.28( \pm )0.24</td>
<td>1.42( \pm )0.40</td>
</tr>
<tr>
<td>DC (nmol/mg protein)</td>
<td>32.9( \pm )2.82</td>
<td>39.4( \pm )14.0</td>
<td>41.4( \pm )14.4</td>
</tr>
<tr>
<td>PC (nmol/mg protein)</td>
<td>2.40( \pm )0.30</td>
<td>2.53( \pm )0.46</td>
<td>2.58( \pm )0.33</td>
</tr>
<tr>
<td>GSH (nmol/mg protein)</td>
<td>4.09( \pm )0.64</td>
<td>4.36( \pm )0.52</td>
<td>2.66( \pm )0.85( ^b )</td>
</tr>
<tr>
<td>MnSOD (U/mg protein)</td>
<td>9.20( \pm )0.75</td>
<td>10.85( \pm )1.2</td>
<td>12.5( \pm )1.21( ^a )</td>
</tr>
<tr>
<td>GSH-Px (nmol/mg protein/min)</td>
<td>211.6( \pm )37.6</td>
<td>247.3( \pm )56.1</td>
<td>250.6( \pm )42.5</td>
</tr>
<tr>
<td>GST (nmol/mg protein/min)</td>
<td>89.3( \pm )68.6</td>
<td>88.6( \pm )18.2</td>
<td>85.4( \pm )13.6</td>
</tr>
</tbody>
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\( a \) \( p<0.05 \) as compared with the control group. \( b \) \( p<0.05 \) as compared with the chronic ethanol group.

**Fig. 1.** Changes in Serum ALT and AST Activities and Hepatic Total Lipid (TL), Triglyceride (TG), Malondialdehyde (MDA) and Diene Conjugate (DC) Levels in Chronic and Chronic Plus Acute Ethanol-Treated Rats (% of Control Values)

ALT (U/l); AST (U/l); TL (mg/g liver); TG (mg/g liver); MDA (nmol/g liver); DC (nmol/g liver). \( a \) Different from the control group, \( p<0.05 \). \( b \) Different from the chronic ethanol group, \( p<0.05 \).

**Fig. 2.** Changes in Hepatic Glutathione (GSH), Vitamin E, Vitamin C Levels and CuZn-Superoxide Dismutase (CuZnSOD), Glutathione Peroxidase (GSH-Px) and Glutathione Transferase (GST) Activities in Chronic and Chronic Plus Acute Ethanol-Treated Rats (% of Control Values)

GSH (\( \mu \)mol/g liver); Vitamin E (nmol/g total lipid); Vitamin C (nmol/g liver); CuZnSOD (U/mg protein); GSH-Px (nmol/mg protein/min); GST (nmol/mg protein/min). \( a \) Different from the control group, \( p<0.05 \). \( b \) Different from the chronic ethanol group, \( p<0.05 \).

On the other hand, in our previous studies, it has been found that MDA and DC levels were increased, GSH levels and GST activities were decreased, and GSH-Px activity re-
mained unchanged in liver homogenates of rats 4 h after a single dose of 5 g/kg of ethanol injection. In addition, we detected no changes in MDA or GSH levels in hepatic mitochondrial fraction 4 h after 5 g/kg of ethanol treatment. In this study, we have evaluated the prooxidant-antioxidant status in rats which received chronic ethanol 4 h after a single dose of 5 g/kg of ethanol. The aim here is to investigate the susceptibility of postalcoholic liver to acute ethanol load. Actually, total lipid, triglyceride, MDA and DC levels increased further, but GSH levels decreased more in the liver homogenates of chronic plus acute ethanol-treated rats compared to the chronic ethanol-treated rats (Fig. 1, 2). Although hepatic vitamin E levels remained unchanged, hepatic vitamin C levels were found to increase in this group compared with the chronic ethanol treated rats. An increase in vitamin C levels may result from increased vitamin C synthesis related to a decrease in GSH levels, as proposed previously. In this study, chronic plus acute ethanol treatment also caused significant decreases in the activities of CuZnSOD, GSH-Px and GST in postmitochondrial fraction compared with chronic ethanol treated rats. These findings pointed out an obvious change in prooxidant-antioxidant balance in the liver of rats following chronic plus acute ethanol treatment. Even under these conditions, no significant differences have been observed in MDA, DC or PC levels, or in GSH-Px and GST activities in hepatic mitochondria following chronic plus acute ethanol treatment (Table 1). However, a decrease in GSH levels and an increase in MnSOD activity were detected in this group. As it is known, CuZnSOD and MnSOD have different responses against increased ROS concentrations. MnSOD, unlike CuZnSOD, is an enzyme which can be induced by its substrate and/or some other factors. However, CuZnSOD activity has been shown to be depressed by ROS. According to this, we can say that an increase in MnSOD activity may play an important role in the regulation of mitochondrial susceptibility against alcohol-induced oxidative stress, inspite of the decrease in GSH.

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