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Effect of type of dietary fat and ethanol on antioxidant enzyme mRNA induction in rat liver

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Abstract We carried out a study to relate the effect of the type of dietary fat and ethanol on antioxidant enzyme mRNA levels in liver in the intragastric feeding rat model. Different types of dietary fat were administered [saturated fat (SE), corn oil (CE) and fish oil (FE)] with ethanol to induce varying severities of liver injury. Ethanol-fed rats were pair-fed with dextrose-fed controls that received isocaloric amounts of dextrose. All animals were killed at 1 month and the following studies were carried out: evaluation of severity of pathologic liver injury, conjugated dienes, and hydrogen peroxide. SE animals had no liver injury. Ethanol-fed rats had severe liver injury, FE animals had moderate liver injury. Ethanol induced GPx mRNA in all dietary groups, with the highest levels seen in the FE group. The pattern of catalase mRNA induction was similar to that of GPx mRNA. In contrast, MnSOD mRNA was decreased compared to controls in animals that developed pathologic liver injury, i.e., CE and FE groups. A positive correlation was seen between conjugated diene levels and GPx mRNA ($r = 0.88$, $P < 0.01$) and catalase mRNA. The similar slopes for the relationship between conjugated dienes and catalase in the fish oil and non-fish oil groups indicate that the same degree of lipid peroxidation increases catalase mRNA to a greater degree in fish oil-fed rats. A positive correlation was also seen between catalase mRNA and H_2O_2 ($r = 0.95$, $P < 0.001$). We propose that the increase in catalase and GPx mRNA levels is probably in response to enhanced lipid peroxidation; the decrease in MnSOD mRNA may predispose the cells to liver injury.

Supplementary key words fatty acids • lipid peroxidation • liver disease catalase • manganese superoxide dismutase • glutathione peroxidase • free radicals

The mechanism(s) contributing to alcohol-induced liver damage remain uncertain. There appears to be increasing evidence that alcohol toxicity may be associated with increased oxidative stress and free radical-associated injury (1–4). Generation of oxygen metabolites such as superoxide ($O_2^-$), hydrogen peroxide, and hydroxyl radicals is believed to be important in the pathogenesis of alcoholic liver injury (5, 6). To counteract these oxidants, cells have several antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (7–9). Eukaryotic cells have two forms of SOD: one found in the mitochondrial matrix, the manganese SOD (MnSOD) and another found predominantly in the cytosol, the copper–zinc SOD (CuZnSOD) (10).

Previous studies examining the effect of chronic ethanol feeding on liver antioxidants have shown decreased mitochondrial glutathione (11, 12), decreased CuZnSOD and GPx activities (13), and increased MnSOD activity (14). The present study was initiated to determine the effects of chronic ethanol administration on the messenger RNA (mRNA) levels of the enzymes involved in antioxidant defense and to relate these changes to development of pathologic liver injury.

To relate antioxidant enzyme mRNA induction to ethanol-induced pathologic changes in the liver, we used the intragastric feeding rat model for alcoholic liver disease (15, 16). This is an extremely useful model in which alterations in mRNA levels can be related to severity of pathologic changes. Based on our previous epidemiologic observations relating the type of dietary fat to alcoholic liver disease (17, 18), we carried out studies using the intragastric feeding model where we demonstrated that animals fed corn oil and ethanol develop pathologic changes in liver whereas animals fed saturated fat are protected (19, 20). Recently, French (21) has shown that feeding fish oil with ethanol produces the most severe liver injury. Thus the use of these three ethanol-feeding models
with different types of dietary fat (saturated fat, corn oil, and fish oil) and their respective controls allowed us to study the relationship between pathologic liver injury and liver antioxidant enzyme mRNAs. As changes in antioxidant defense occur in response to lipid peroxidation, we also related the mRNA levels for these enzymes to lipid peroxidation measurements.

MATERIALS AND METHODS

Animals

The experimental animals were male Wistar rats weighing between 225 and 230 g. Six groups of rats (three rats per group) were studied: saturated fat (SD), saturated fat + ethanol (SE), corn oil + dextrose (CD), corn oil + ethanol (CE), fish oil + dextrose (FD), and fish oil + ethanol (FE). All animals were killed at 1 month and the following studies were carried out: evaluation of pathological changes in the liver, microsomal conjugated dienes and H₂O₂, and mRNA levels for glutathione peroxidase, catalase, and manganese superoxide dismutase.

All animals were pair-fed by continuous infusion of liquid diets through permanently implanted gastric tubes as described previously (15). The percentage of calories derived from fat was 25% of total calories. The fatty acid composition of the diets is shown in Table 1. Proteins, carbohydrates, minerals, and vitamins were administered as described previously (15). The percentage composition of diets was such that the animals in all dietary groups consumed the same amount of calories, proteins, and vitamins. All control animals were pair-fed the same diet as ethanol-fed rats except that ethanol was isocalorically replaced by dextrose. All diets were prepared fresh daily. In particular, the fish oil diet was stored in air-tight containers, filled with nitrogen, in a cold room at 4°C. Malondialdehyde content of the diets was < 5 nmol/g of sample suggesting that very little autooxidation was taking place. The amount of ethanol was initially started at 8 g/kg per day and gradually increased as tolerance developed. Blood alcohol levels were maintained between 150 and 350 mg/dl. All animals were killed at 1 month after the start of feeding. All animals received humane care in compliance with the National Institutes of Health criteria for care of laboratory animals.

Histologic analysis

A small sample of liver was obtained at the time of killing and formalin-fixed. Hematoxylin and eosin stain was 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 (16): steatosis (the percentage of liver cells containing fat): 1+ = < 25% of cells containing fat, 2+ = 26-50%, 3+ = 51-75%, 4+ = > 75%; inflammation and necrosis: one focus/lodule = 1+, two or more foci/lobule = 2+.

RNA isolation and Northern blot analysis

RNA was isolated from liver according to the procedure described by Chomczynski and Sacchi (22) and then fractionated on 1% agarose gel containing 3% formaldehyde, 0.02 M MOPS pH 7.4 (3-[N-morpholino]propanesulfonic acid) and 0.001 M disodium EDTA (ethylenediaminetetraacetic acid). The RNA was visualized by ethidium bromide staining and then transferred to nitrocellulose overnight with 10 × SSC (standard saline citrate, pH 7.0). GPx mRNA levels were determined using the 0.55 kb fragment of the human cDNA GPx containing codon 92 and the 3’ untranslated region of glutathione peroxidase (23). Catalase gene expression was determined using a 1.9 kb insert from the cDNA pH CAT 1.2 (kind gift from Dr. G. Mullenbach) (24). MnSOD mRNA was detected using a 0.88 kb insert from the cDNA pH SOD α2 (ATCC 39786). Hybridization with labeled probes (32P-labeled ATP) was done for 24 h at 42°C in 50% formamide, 2 × SSC (1 × SSC = 150 mmol/l NaCl, 15 mmol/l sodium citrate, pH 7.0) 5 × Denhardts, 0.1% sodium dodecyl sulfate (SDS); 10% dextran sulfate (Sigma Co., St. Louis, MO). The various mRNA were quantified by autoradiography. The autoradiograms were scanned using laser densitometry. Rat α-tubulin oligonucleotide ON-50 (25) was used as a control mRNA probe to verify RNA load consistency and RNA integrity. The tubulin mRNA is generally unresponsive to the treatments used in this study. The results were expressed as a multiple of the mean value seen in the saturated fat plus dextrose group.

TABLE 1. Fatty acid composition (percentage by weight) of saturated fat, corn oil, and fish (menhaden) oil diets

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Saturated Fat</th>
<th>Corn Oil</th>
<th>Fish (Menhaden) Oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Octanoic (C8:0)</td>
<td>52.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Decanoic (C10:0)</td>
<td>33.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lauric (C12:0)</td>
<td>0.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Myristic (C14:0)</td>
<td>0</td>
<td>0.1</td>
<td>11.6</td>
</tr>
<tr>
<td>Palmitic (C16:0)</td>
<td>1.4</td>
<td>11.3</td>
<td>13.0</td>
</tr>
<tr>
<td>Palmitoleic (C16:1 n-9)</td>
<td>0</td>
<td>0</td>
<td>13.3</td>
</tr>
<tr>
<td>Stearic (C18:0)</td>
<td>1.0</td>
<td>1.9</td>
<td>2.1</td>
</tr>
<tr>
<td>Oleic (C18:1 n-9)</td>
<td>1.0</td>
<td>25.5</td>
<td>6.7</td>
</tr>
<tr>
<td>C18:1 n-7</td>
<td>0</td>
<td>0</td>
<td>3.3</td>
</tr>
<tr>
<td>Linoleic (C18:2 n-6)</td>
<td>1.5</td>
<td>59.0</td>
<td>1.1</td>
</tr>
<tr>
<td>Arachidonic acid (C20:4 n-6)</td>
<td>0</td>
<td>0</td>
<td>0.7</td>
</tr>
<tr>
<td>Eicosapentaenoic acid (C20:5 n-3)</td>
<td>0</td>
<td>0</td>
<td>1.9</td>
</tr>
<tr>
<td>Docosahexaenoic acid (C22:6 n-3)</td>
<td>0</td>
<td>0</td>
<td>8.2</td>
</tr>
</tbody>
</table>

TABLE 2. Pathological changes in the different treatment groups

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Animal No.</th>
<th>Fatty Liver</th>
<th>Necrosis</th>
<th>Inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn oil plus ethanol</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Fish oil plus ethanol</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

*None of the dextrose-fed controls or animals fed saturated fat and ethanol developed pathological changes.

Measurement of microsomal conjugated dienes and hydrogen peroxide

Microsomes were prepared from the liver as previously described (26). Lipid was extracted according to the method of Bligh and Dyer (27) and conjugated dienes were measured by the method of Recknagel and Glende (28). Hydrogen peroxide was determined by measuring the formation of formaldehyde from methanol (29). Reactions were carried out in 200 mM KCl, 50 mM Tris-HCl, pH 7.4, 100 mM methanol, 300 units of catalase, and 0.5 mg of microsomal protein in a final volume of 100 µl. Reactions were initiated by addition of NADPH (1.2 mM) and terminated by the addition of 30 µl 20% trichloracetic acid. The generation of formaldehyde was assayed by the Nash reaction (30).

Statistics

All results are expressed as mean ± SE except where indicated. Comparison between groups was done using analysis of variance followed by Tukey’s multiple range test. Pearson’s correlation coefficient r was used to evaluate associations.

RESULTS

In each of the groups studied, the rats increased their body weight at a constant rate. Although the average weight gain was lower in the ethanol-fed rats compared to the respective dextrose-fed controls, the differences between the various groups were not significant. The blood alcohol levels (mg/dl) (mean ± standard error) in the ethanol-fed groups were: SE 242 ± 26, CE 223 ± 35, FE 217 ± 31. The differences between groups were not significant. The pathologic changes in the animals in the various groups are shown in Table 2. The most severe pathology was seen in the fish oil-ethanol (FE) group (pathology score range 6–8) (Fig. 1), moderate to severe changes (pathology score range: 3–5) were seen in the corn oil-ethanol (CE) group (Fig. 2), and no pathologic changes were seen in the saturated fat-ethanol group (Fig. 3). None of the dextrose-fed control animals developed pathologic changes. We examined the changes in the antioxidant enzyme mRNA levels in the various groups. A
Fig. 4. Northern blot analysis of hepatic antioxidant messenger RNAs. Total RNA was extracted from snap-frozen liver tissue and subjected to Northern blot analysis as described in Materials and Methods. Blots were probed with *32*P-labeled cDNA probes to glutathione peroxidase, catalase, and manganese superoxide dismutase. Representative Northern blots are shown in panels A to D. Panel A shows ethanol-related increases in GPx mRNA in each dietary group. A similar pattern is seen for catalase mRNA (panel B). The lowest level for MnSOD mRNA is seen in the group of animals developing severe pathologic liver injury (FE). Uniformity of lane to lane loading was confirmed by inspection of the ethidium bromide-stained gel (panel D). Normalization of the antioxidant mRNA signals to the tubulin signal was carried out for each group. The mean ± standard error for the levels of antioxidant mRNAs (arbitrary units) is shown in Figs. 5-7. SD, saturated fat and dextrose; SE, saturated fat and ethanol; CD, corn oil and dextrose; CE, corn oil and ethanol; FD, fish oil and dextrose; FE, fish oil and ethanol.

A typical Northern blot pattern for the antioxidant mRNAs in the various groups is shown in Fig. 4. All of the mRNA transcript levels appeared to be regulated by both the type of dietary fat and ethanol. Figure 5 shows that the lowest levels of GPx mRNA were seen in the SD group and highest levels were seen in the FE group. In each dietary group, ethanol caused a further increase in GPx transcript levels. The pattern for diet and ethanol-related changes for catalase mRNA was similar to that seen with GPx. The highest level of catalase mRNA in the dextrose-
f fed control groups was seen in the FD group and the lowest level was seen in the SD group (Fig. 6). Thus, the degree of saturation of dietary fatty acids appeared to determine the level of catalase mRNA. As seen with GPx, in each dietary group, the level of catalase mRNA was further increased by ethanol. The changes in MnSOD mRNA were different compared to those seen with catalase and GPx (Fig. 7). Ethanol feeding did not change MnSOD mRNA levels in rats fed saturated fat; in rats fed both corn oil and fish oil, a decrease in MnSOD transcript levels was seen. The lowest level of MnSOD mRNA was seen in the FE group.

Because the changes in the above antioxidant enzyme mRNAs were probably occurring in response to changes in levels of lipid peroxides, we correlated changes in various mRNA levels with microsomal conjugated diene levels. Catalase mRNA was also correlated with microsomal H2O2. A significant correlation (r = 0.91, P < 0.01) was seen between the degree of lipid peroxidation (A232) and GPx mRNA (Fig. 8). A significant correlation was also seen between A232 and catalase mRNA levels (Fig. 9). However, there are two different responses for catalase mRNA in the various dietary groups. The increase in catalase mRNA for a given level of lipid peroxidation was higher in the fish oil group (r = 0.91, P < 0.01) than in the corn oil and saturated fat groups (r = 0.91, P < 0.01). A significant correlation (r = 0.95, P < 0.01) was also seen between catalase mRNA and microsomal H2O2 concentration (Fig. 10). There was no significant correlation between MnSOD mRNA levels and either A232 or hydrogen peroxide.

**DISCUSSION**

The results of our study show that changes in mRNA levels for glutathione peroxidase, catalase, and MnSOD...
were related to type of dietary fat which in turn was related to the severity of liver injury in the intragastric feeding model. Thus the diet-related alterations in the mRNAs for antioxidant enzymes may play an important role in the development of liver injury.

The prevention of excess free radical formation is a vital first step for cell survival. The major antioxidant enzyme systems available to cells during oxidant stress include catalase, superoxide dismutase, and glutathione peroxidase (7-9). Catalase is a 240,000 molecular weight tetrameric hemoprotein that catalyzes the dismutation reaction: $2\text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} + \text{O}_2$. Catalase, which is located mainly in the peroxisomes, has reductive activity mainly for small molecules rather than lipid hydroperoxide products of lipid peroxidation (31, 32). The other key enzyme that has overlapping capacity for the reduction and elimination of $\text{H}_2\text{O}_2$ is glutathione peroxidase (GPx) (33). GPx exists predominantly in the cytosol (33) and is the key enzyme in the glutathione redox cycle. GPx complements catalase as a reducing system for $\text{H}_2\text{O}_2$ but exceeds the ability of catalase to eliminate other types of toxic intracellular hydroperoxides (34). GPx is considered the preferential pathway of elimination of low concentrations of $\text{H}_2\text{O}_2$. Catalase has a low affinity but high capacity for $\text{H}_2\text{O}_2$ so it becomes more important as rates of $\text{H}_2\text{O}_2$ generation are increased. This is clearly suggested by the correlation between $\text{H}_2\text{O}_2$ production and catalase mRNA levels.

The increase in mRNA levels for both GPx and catalase in relation to the degree of unsaturation of the type of dietary fat is consistent with the observations of other investigators who showed that the type of dietary fat affects antioxidant enzyme activities in various organs (35, 36). Part of this increase in mRNA levels in our study can be explained by the fact that $\text{H}_2\text{O}_2$ can be generated at high rates via peroxisomal $\beta$-oxidation of fatty acids and that the magnitude of the stimulation of peroxisomal $\beta$-oxidation is dependent on the chain length of the fatty acid (37, 38). Furthermore, Demoz, Willumsen, and Berge (39) showed that the increase in peroxisomal $\beta$-oxidation in rat liver seen with feeding of $n-3$ fatty acids is associated with increased glutathione peroxidase and catalase activity. Misra et al. (40) showed that, in perfused rat livers, ethanol feeding increases both $\text{H}_2\text{O}_2$ generation and catalase activity. Our observation that ethanol further
enhances catalase mRNA and that the highest levels are seen in rats fed fish oil and ethanol is consistent with a role for both ethanol and fatty acids in enhancing H$_2$O$_2$ production by increasing acyl-CoA oxidase activity (40, 41).

Hydrogen peroxide is a precursor of a number of oxygen-derived radicals including hydroxyl radicals (42). Thus our observation that the levels of both H$_2$O$_2$ and lipid peroxidation (conjugated dienes) correlated with catalase mRNA levels may represent a compensatory response to increased production of H$_2$O$_2$ and lipid peroxides. This is further suggested by the different slopes of correlation seen in the fish oil- and non-fish oil-treated groups because feeding the highly unsaturated n-3 fatty acids, even without ethanol, would be expected to result in greater production of H$_2$O$_2$ (43).

The correlation between GPx mRNA levels and conjugated dienes probably also reflects a compensatory response to increased oxidative stress. Previous studies by Hirano et al. (12) have demonstrated a progressive and selective decline of mitochondrial glutathione in the intragastric feeding rat model. Glutathione is a substrate for glutathione peroxidase. It is believed that reduced glutathione (GSH) is one of the most important protective factors against oxidative damage (44) and that GSH-dependent enzymes such as GPx may, in addition to GSH, act as free radical scavengers (45). The efficiency of GPx in protecting the cell against free radical species is well documented (46). Thus the increase in GPx mRNA may be a protective response to overcome decreased liver GSH concentrations described in this model. Also, the relationship between the type of dietary fat and GPx activity has been previously demonstrated (35, 36) and is believed to be a response to the increased oxidative stress seen with administration of unsaturated fatty acids (43).

It is generally thought that MnSOD scavenges O$_2^-$ generated at the NADH-Q and QH$_2$-cytochrome C segments of the respiratory chain in the mitochondria (10). Mitochondrial respiratory control is impaired after ethanol feeding and is believed to be associated with production of O$_2^-$ and H$_2$O$_2$ (47). The most dramatic ethanol-related alterations in mitochondria include a 50–60% decrease in the activity and heme content of cytochrome oxidase and 30–40% decrease in electron transport and proton translocation through the NADH–ubiquinone reductase portion of the electron transport chain (48). These changes lead to lower rates of ATP synthesis in the mitochondria. Because glutathione is not synthesized in the mitochondria (II) and mitochondria do not contain catalase to cope with H$_2$O$_2$ produced by aerobic respiration, MnSOD becomes extremely important in protecting mitochondria against increased oxidative stress.

The decreased mRNA levels of MnSOD were seen only in the groups of rats developing pathologic liver injury (corn oil and fish oil plus ethanol). The decrease was greater in the fish oil–ethanol group which showed the most severe liver injury. Although the mechanism(s) leading to a decreased level of MnSOD mRNA in association with feeding ethanol and corn oil and fish oil are unknown, this finding is extremely important in view of the known relationship between MnSOD and tumor necrosis factor (TNF) cytotoxicity (49, 50). The relationship between TNF cytotoxicity and cellular MnSOD mRNA levels has been clearly demonstrated by several investigators (49–51) who showed that cells expressing increased mRNA for MnSOD are resistant to TNF cytotoxicity whereas cells with lower expression of MnSOD were extremely sensitive to TNF-mediated cytotoxic effects. TNF plays an important role in the pathogenesis of both experimental and clinical alcohol liver disease (52–54).

Cytotoxicity of TNF can be mediated through induction of oxidative radicals such as O$_2^-$, H$_2$O$_2$, and $^•$OH (55). We have recently shown that TNF mRNA is increased in corn oil–ethanol-fed rats (liver injury present) and not in rats fed saturated fat and ethanol (no liver injury) (56). Thus the lower MnSOD mRNA levels in the CE and FE groups may provide one possible explanation for the enhanced severity of liver injury in these groups. TNF mRNA levels in liver are higher in rats fed fish oil and ethanol compared to corn oil–ethanol-fed rats (Nanji, A. A., unpublished observations). Although the induction of TNF mRNA does not necessarily correlate with increased TNF protein levels or activity, our previous studies have shown that TNF production by hepatic non-parenchymal cells in corn oil–ethanol-fed rats is greater than the TNF production in rats fed saturated fat and ethanol (57). Plasma TNF is not normally detectable in rats chronically fed ethanol unless endotoxin is also administered at the same time (58).

A decrease is MnSOD activity has also been observed in rats in which oxidative stress was induced by suprarehepatic vena cava occlusion and reperfusion (59). The marked decrease in MnSOD activity is associated with increased mitochondrial H$_2$O$_2$ generation. Thus the increased oxidative stress described in the CE and FE rats combined with the observed decrease in mitochondrial glutathione (12) and absence of catalase in mitochondria could contribute to the decrease in MnSOD mRNA. Malis et al. (60) have shown that mitochondrial membranes from rats fed n-3 fatty acids are more susceptible to phospholipase A$_2$-mediated injury than mitochondrial membranes isolated from rats fed saturated fat. These investigators found a greater impairment of mitochondrial respiration in the fish oil-fed group. Other investigators (61) have also shown impaired mitochondrial function, e.g., uncoupled respiratory rates in mitochondria exposed to peroxidized polyunsaturated fatty acids. Thus, the association between presence of liver injury and decreased MnSOD mRNA may be related to loss of mitochondrial membrane integrity and subsequent cell death.

In summary, the results of our study indicate that GPx...
and catalase mRNA levels are increased by ethanol feeding in all dietary groups and that both enzyme mRNAs are also regulated by the type of dietary fat. These increases in mRNA levels, which correlated with lipid peroxidation, suggest that the increased levels for these mRNAs may be a compensatory response to oxidative stress. In contrast, increased levels of MnSOD mRNA occur in the dietary groups that develop pathologic liver injury. The enhanced sensitivity of cells with decreased MnSOD mRNA to TNF and reactive oxygen species may be an important contributing factor in the pathogenesis of alcoholic liver disease.

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