Lipid Peroxidation, CYP2E1 and Arachidonic Acid Metabolism in Alcoholic Liver Disease in Rats\textsuperscript{1,2}

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ABSTRACT The role of cytochrome P450 metabolism of fatty acids and lipid peroxidation in the alterations of the fatty acid composition of the liver and liver pathology was investigated. The CYP2E1 inhibitors partially prevented CYP2E1 induction by ethanol and completely blocked lipid peroxidation. However, the liver pathology induced by ethanol was only partially prevented as was the decrease in arachidonic acid in total liver lipid, triglycerides and cholesterol esters. This means that liver peroxidation induced by ethanol can not completely account for the liver pathology or the decrease in arachidonic acid caused by ethanol. Lauric acid \(\omega-1\) hydroxylation by the liver microsomes in vitro was increased by ethanol and partially blocked by CYP2E1 inhibitors. However, although ethanol feeding increased the total hydroxylation and epoxidation of arachidonic acid, these were not inhibited by CYP2E1 inhibitors. Thus the ethanol-induced arachidonic acid depletion is not likely due to CYP2E1 metabolism of arachidonic acid, since the severity of liver pathology correlated negatively with the decrease in arachidonic acid in the ethanol-fed rats. The increase in its metabolism by microsomes and decrease in synthesis may be an important mechanism of ethanol-induced liver injury. J. Nutr. 127: 907S–911S, 1997.

KEY WORDS: • cholesterol • cytochrome P450 • ethanol • fatty acids • rats

Previous studies have indicated that lipid peroxidation resulting from free radical generation is caused by ethanol metabolism by the liver (Nordmann et al. 1992). However, when we recently attempted to establish a relationship between lipid peroxidation and the presence of liver disease in ethanol-fed rats, we found that lipid peroxidation could account for only a part of the disease. For instance, feeding cytochrome P450 2E1 (CYP2E1)\textsuperscript{*} inhibitors decreased liver microsomal lipid peroxidation to levels below the dextrose control levels (Morimoto et al. 1993 and 1995a), yet when the liver pathology was semiquantitated in the livers of these groups of rats the pathology was only partially ameliorated by these inhibitors (Morimoto et al. 1993 and 1995a). Likewise, the CYP2E1 inhibition was only partially effective in reducing ethanol induction of CYP2E1 (Morimoto et al. 1993 and 1995a). The high fat, low carbohydrate diet used in these studies may also influence the degree of liver pathology and lipid peroxidation possibly, because ethanol induction of the cytochrome P450 system is reduced if the diet is rich in carbohydrates (Nanajima et al. 1992, Yonekura et al. 1993, Yoo et al. 1991).

We further studied the role of lipid peroxidation in the reduction of arachidonic acid [20:4(n-6)] in the livers of the same ethanol-fed rats (Morimoto et al. 1993, 1995a and 1995b) used to study the fatty acid composition of liver lipids. We and others had previously shown that ethanol feeding reduced hepatic levels of 20:4(n-6) (French et al. 1970, Reitz 1993). We measured levels of the fatty acids in the total liver lipid fraction and found that ethanol feeding markedly reduced the percentage of 20:4(n-6); CYP2E1 inhibitors only partially inhibited this change. Thus, lipid peroxidation by CYP2E1 could not totally account for the decrease in 20:4(n-6).

Other explanations for the reduced levels of 20:4(n-6) in liver lipids include ethanol inhibition of \(\Delta_5\) and \(\Delta_6\) desaturases, which are responsible for the conversion of linoleic acid [18:2(n-6)] to 20:4(n-6) (Reitz 1993). In the present article we explore the possible role that liver microsomal fatty acid metabolism of 20:4(n-6) might play in the turnover of 20:4(n-6) stimulated by P450 isomers induced by ethanol. We also investigated the changes in cholesterol ester fatty acids to see whether they participate in the ethanol-induced reduction in...
20:4(n-6). We found previously (Morimoto et al. 1995b) that CYP2E1 induction by ethanol feeding correlated inversely with the liver 20:4(n-6) levels (r = -0.77, P < 0.002). Therefore, we studied the in vitro metabolism of 20:4(n-6) by liver microsomes from rats fed ethanol with or without CYP2E1 inhibitors.

METHODS

Animals and diets. Male Wistar rats weighing 230 to 290 g (Charles River, Hollister, CA) were surgically implanted with a permanent intragastric cannula. The procedures were approved by the Research and Education Institute Animal Care Committee in accordance with the guidelines for animal care as described by the Academy of Science (1996). The rats were fed a liquid diet with ethanol or isocaloric dextrose with or without CYP2E1 inhibitors diallyl sulphone (DAS) [200 mg/(kg·d)] or phenethyl isothiocyanate (PIC) [1 mmol/(kg·d)] for 1 mo. A high blood alcohol level (45–68 mmol/L) was maintained using a 80 g/L solution of ethanol. The diet solution was given at a rate of 750 kcal/(kg·d). The amount of liquid diet was adjusted according to the body weight of the alcohol-fed rats as determined weekly. The mean energy derived from ethanol or dextrose has 325 ± 9.5 kcal/(kg·d). The initial diet administered included 6 g/(kg·d) of ethanol, with 19.2% of energy derived from ethanol, 40.8% from fat, 31.8% from protein and 8.2% from carbohydrate (Fig. 1). The ethanol was increased gradually (on the basis of the blood alcohol level) to a level of 14 g/(kg·d), with 35.7% of energy derived from ethanol, 6.6% from carbohydrate, 32.5% from fat and 25.3% from protein.

Liquid diets were prepared fresh each day as described previously (Morimoto et al. 1995b) with lactalbumin hydrolysate as the protein source, corn oil as the fat source and dextrose as the carbohydrate source. The vitamin and salt mixtures used were those of the AIN-76A diet (Dyets, Bethlehem, PA; 2.65 g/L and 9.27 g/L, respectively). The diet was enriched with L-threonine (1.05 g/L) and choline (0.53 g/L). The suspending agent K from Bio-Serve (Frenchtown, NJ) was used to prevent settling. Non-nutrient fiber (Alphaceal, IGN Biochemicals, Cleveland, OH) and water were provided in unrestricted amounts.

**Cholesterol ester fatty acid composition.** Liver lipids were extracted according to the method of Bligh and Dyer (1959). The protein in the homogenate was measured by the Bradford (1976) method. Cholesterol esters were separated by TLC, and the TLC scappings were methylated. An internal standard of 19:0 free fatty acid was added directly to the cholesterol ester spot on the TLC plate before scraping and methylatation. The fatty acid methyl esters were extracted with petroleum ether, dried and rechromatographed on TLC to remove free cholesterol. The methyl esters were extracted with chloroform, dried and redissolved in carbon disulfide. The samples were analyzed by gas chromatography on a SP-2340 capillary column in a Shimadzu gas chromatograph 14A (Columbia, MD) (Morimoto et al. 1995b).

**Liver microsomal metabolites of lauric and arachidonic acid.** Liver microsomes were prepared and incubated with radiolabeled lauric or arachidonic acid (4°C) with a NADPH-generating system, and the metabolites were separated and quantified using reverse-phase HPLC resolution as described by Laethem et al. (1993). The major metabolites analyzed included ω and ω-1 hydroxylated lauric acid and arachidonic acid, ω, ω-1 and ω-2 hydroxylated eicosatetraenoic acids (HETE), and epoxyeicosatrienoic acid (EET) 5-6, 8-9, 11-12, 14-15-EET. Total arachidonic acid metabolites were calculated.

**Biochemical analysis.** Chlordiazepoxide hydroxylation by liver microsomes was measured using a HPLC procedure (Lucas et al. 1993). **Statistical analysis.** The data were statistically analyzed by one-way ANOVA and pairwise multiple comparisons (Bonferroni's test).

Correlations were performed by linear regression analysis using Sigma Stat and Sigma Plot software (Jandel Scientific, San Francisco, CA). Differences were considered significant if P < 0.05.

**RESULTS**

**Fatty acid metabolism by hepatic microsomes in vitro.** Table 1 summarizes the data on microsomal metabolism of radiolabeled lauric and arachidonic acids using data from the four treatment groups. The ethanol-fed rats showed significantly increased ω-1 hydroxylation of lauric acid, and this change was partially but not significantly inhibited by CYP2E1 inhibitors. Hydroxylation of chlordiazepoxide as a measure of CYP2E1 activity (r = 0.875, P < 0.001) was markedly increased by ethanol, and this was partially inhibited by the inhibitors of CYP2E1 (Fig. 2). The rate of ω-1 hydroxylation and chlordiazepoxide hydroxylation, total P450, and CYP2E1 levels were positively correlated with ω-1 hydroxylation of lauric acid (r = 0.963, P < 0.001; r = 0.974, P < 0.001; and r = 0.862, P < 0.001, respectively). A similar correlation was found for ω-hydroxylation and total P450 (r = 0.798, P < 0.001). Because CYP2E1 does not catalyze the ω hydroxylation of lauric acid, this correlation must reflect the coordinated induction of another isofrom, presumably a P450, and CYP2E1 levels were positively correlated with the inability of CYP2E1 to metabolize at this position (Table 1).

There was no significant increase in the microsomal formation of the ω, ω-1 or ω-2 hydroxylated metabolites of 20:4(n-6) (Table 1). However, there was a significant increase in the formation of epoxide metabolites that was not inhibited by PIC or DAS treatment (Table 1), which indicates that isoforms other than CYP2E1 were responsible for this increase in metabolism. When the 20:4(n-6) metabolites in Table 1 were totaled, a significant increase was noted in the ethanol-fed rats (Fig. 3), and this was unaffected by the CYP2E1 inhibitors, indicating that P450 isoforms other than CYP2E1 were involved. The reduction in 20:4 was negatively correlated with the formation of epoxide metabolites such as 14,15-EET (r = -0.838, P < 0.001), 11,12-EET (r = -0.868, P < 0.001)
### TABLE 1

Liver microsomal fatty acid hydroxylase metabolites formed: Effect of the CYP2E1 inhibitors diallyl sulfoxide (DAS) and phenethyl isocyanate (PIC) on ethanol-induced cytochrome P450

<table>
<thead>
<tr>
<th>Substrate and metabolites</th>
<th>Dietary group</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Ethanol</td>
<td>Ethanol + DAS</td>
<td>Ethanol + PIC</td>
<td></td>
</tr>
<tr>
<td>Lauric acid</td>
<td>190.7 ± 70.7</td>
<td>3870 ± 1429*</td>
<td>2062 ± 619</td>
<td>2195 ± 480</td>
<td></td>
</tr>
<tr>
<td>ω-1 19-HETE</td>
<td>197.7 ± 143.7</td>
<td>1418 ± 461*</td>
<td>1048 ± 372</td>
<td>1720 ± 600*</td>
<td></td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>1.3 ± 0.6</td>
<td>4.7 ± 2.2</td>
<td>4.3 ± 0.5</td>
<td>4.6 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>ω-1 19-HETE</td>
<td>1.6 ± 0.6</td>
<td>3.7 ± 1.4</td>
<td>4.5 ± 1.2</td>
<td>4.5 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>β 14,15-EET</td>
<td>1.7 ± 1.0</td>
<td>3.1 ± 1.5</td>
<td>3.4 ± 0.8</td>
<td>3.3 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>11,12-EET</td>
<td>3.6 ± 0.1</td>
<td>10.5 ± 2.6*</td>
<td>12.5 ± 2.0*</td>
<td>11.9 ± 1.3*</td>
<td></td>
</tr>
<tr>
<td>8,9-EET</td>
<td>2.7 ± 0.5</td>
<td>7.5 ± 1.0</td>
<td>7.5 ± 1.2</td>
<td>7.4 ± 0.3*</td>
<td></td>
</tr>
<tr>
<td>6,5-EET</td>
<td>0.8 ± 0.3</td>
<td>1.1 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>1.1 ± 0.4</td>
<td></td>
</tr>
</tbody>
</table>

1 Values are means ± SD; n = 3 or 4. *Significantly different from control [P < 0.05, one-way ANOVA and pairwise multiple comparisons (Bonferroni’s t test)]. HETE = hydroxidated ercosatetraenoic acid, EET = epoxyeicosatrienoic acid.

Changes in cholesterol ester fatty acids. The changes in total cholesterol fatty acids in ethanol-fed rats, with or without CYP2E1 inhibitor treatment, are shown in Table 2. Ethanol increased the total cholesterol fatty acids by fourfold.

Ethanol feeding did not change 18:0 levels (Table 2), whereas PIC caused a significant elevation, suggesting that PIC may inhibit the conversion of 18:0 to 18:1 by Δ9 desaturase. Oleic acid (18:1) was also reduced by PIC (Table 2), which further supports this conclusion. Consequently PIC decreased the 18:1/18:0 ratio. Ethanol had no significant effect on this ratio. We conclude that ethanol does not affect Δ9 desaturase activity, nor does CYP2E1 induction by ethanol affect the 18:1/18:0 ratio.

Ethanol feeding increased 18:2 and decreased 20:4. Phenethyl isothiocyanate prevented the increase in 18:2 but did not affect the ethanol-induced decrease in 20:4, suggesting that CYP2E1 induction by ethanol was not the cause for the reduction in 20:4, but rather inhibition of Δ5 and Δ6 desaturase by ethanol accounts for the decrease in 20:4, which is negatively correlated with the severity of the liver pathology (r = −0.622, P < 0.003). This would suggest that and 8,9-EET (r = −0.879, P < 0.001). Thus increases in epoxide formation that may occur on release of 20:4(n-6) may contribute to the decrease in 20:4(n-6) in the total lipid pool during ethanol treatment.

**FIGURE 2** Bar graph showing that the hydroxidation of chlorzoxazone (CZX) was markedly increased by ethanol feeding (P < 0.05 compared with control). The inhibitors of CYP2E1 reduced the level of hydroxidation stimulated by ethanol feeding. Cont. = control; ETOH = ethanol; DAS = diallyl sulfide; PIC = phenethyl isothiocyanate.

**FIGURE 3** Total 20:4(n-6) metabolites listed in Table 1 were significantly increased (P < 0.05) by liver microsomes in rats fed ethanol with or without inhibitors. Cont. = control; ETOH = ethanol; DAS = diallyl sulfide; PIC = phenethyl isothiocyanate.
TABLE 2
Cholesterol ester fatty acid composition of the liver: Effect of the CYP2E1 inhibitor Phenethyl Isothiocyanate (PIC) in rats fed ethanol intragastrically

<table>
<thead>
<tr>
<th>Dietary group</th>
<th>Total fatty acids, nmol/mg protein</th>
<th>Individual fatty acids, %</th>
<th>Fatty acid ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dextrose</td>
<td>Ethanol</td>
<td>Ethanol + PIC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.29 ± 0.35</td>
<td>6.54 ± 1.872</td>
<td>6.40 ± 3.62</td>
</tr>
<tr>
<td>Total fatty acids, nmol/mg protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Individual fatty acids, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>17.8 ± 2.8</td>
<td>15.1 ± 2.2</td>
<td>20.8 ± 7.4 33.0 ± 7.62</td>
</tr>
<tr>
<td>18:0</td>
<td>4.5 ± 1.5</td>
<td>3.1 ± 0.5</td>
<td>6.9 ± 3.43 13.9 ± 3.42</td>
</tr>
<tr>
<td>18:1</td>
<td>35.4 ± 4.1</td>
<td>37.5 ± 2.2</td>
<td>38.8 ± 4.9 24.9 ± 4.62</td>
</tr>
<tr>
<td>18:2</td>
<td>31.4 ± 7.8</td>
<td>40.8 ± 1.62</td>
<td>33.3 ± 5.93 16.0 ± 4.32</td>
</tr>
<tr>
<td>20:4</td>
<td>5.5 ± 1.0</td>
<td>3.8 ± 1.12</td>
<td>2.9 ± 0.72 6.2 ± 0.9</td>
</tr>
<tr>
<td>Fatty acid ratios</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:1/18:0</td>
<td>8.8 ± 3.4</td>
<td>12.8 ± 3.7</td>
<td>7.8 ± 4.9 1.9 ± 0.43</td>
</tr>
<tr>
<td>20:4/18:2</td>
<td>0.16 ± 0.05</td>
<td>0.09 ± 0.03</td>
<td>0.09 ± 0.04 0.4 ± 0.1</td>
</tr>
</tbody>
</table>

1 Values are means ± so, n = 6 or 7.
2 P < 0.05 compared with control.
3 P < 0.05 compared with ethanol.

The reduction of 20:4 may be one mechanism of ethanol-induced liver injury.

DISCUSSION

The results reported here add additional evidence to the understanding of the role of CYP2E1 induction by ethanol in the pathogenesis of alcoholic liver disease (Fig. 4). CYP2E1 may alter the lipid composition of the liver through free radical attack (Nordmann et al. 1992, Morimoto et al. 1995a and 1995b, French et al. 1993), by a protein adduct necantigen autoimmune mechanism (Albano et al. 1996) or via free fatty acid metabolism. In the present article we focused on the changes in arachidonic acid, which was decreased in the total fatty acid (Morimoto et al. 1995b), triglyceride fatty acid and cholesterol ester fatty acid pools.

We found that CYP2E1 inhibitors did not block the ethanol-induced decrease in cholesterol ester fatty acid 20:4(n-6) or the in vitro hydroxylation and epoxidation of 20:4(n-6), which negates the role that CYP2E1 might play in reducing 20:4(n-6) in the hepatic lipid pools. Instead, the in vitro metabolism by hepatic microsomes was increased by the induction by ethanol of other isoforms of P450 not measured in this study. For instance, ethanol feeding induces an increase in P450-4A1 (Ma et al. 1993); CYP2B1 and 2B2 are induced by ethanol feeding (Nanji et al. 1994, Takahashi et al. 1992), and CYP3A is induced in liver cells in culture by ethanol (Kostrubsky et al. 1996). Roberts et al. (1995) reported that CYP1A1, CYP2B1 and CYP3A1 activities were induced by ethanol.

Thus at least four isoforms may be induced by ethanol ingestion. The total P450 is doubled by ethanol ingestion, and CYP2E1 constitutes a major fraction of this increase (French et al. 1993, Morimoto et al. 1993 and 1995a).

The use of mechanism-based inhibitors of CYP2E1 such as PIC and DAS (Brady et al. 1991, Koop 1992, Pan et al. 1993) in this study proved useful in discriminating between the metabolism of free 20:4(n-6) by liver microsomal CYP2E1 and by other P450 isoforms in vitro. Neither hydroxylation nor epoxidation of 20:4 was inhibited by feeding PIC or DAS. However, interpretation of the effects of these inhibitors is complicated because these inhibitory compounds also inhibit CYP2E1 gene expression and, in the case of DAS, induce gene expression of other P450 isoforms such as 2B1/2 (Pan et al. 1993).

It was anticipated that the induced CYP2E1 in liver microsomes was responsible in part for the hydroxylation of 20:4(n-6) in vitro, because rabbit CYP2E1 metabolized arachidonic acid to two major metabolites, 19-hydroxyeicosatetraenoic acid (19-HETE) and ω-2 hydroxylated metabolite (18-HETE), in a reconstituted system (Laethem et al. 1993). A smaller percentage of the metabolites derived from CYP2E1 metabolism of 20:4(n-6) were epoxyeicosatrienoic acids (EET), i.e., 14,15-EET, 11,12-EET and 8,9-EET. In the present study, epoxides (EET) were the predominant metabolites. None of these metabolites formed were reduced by the CYP2E1 inhibitors.

The interest in the formation of these 20:4 metabolites is in their potential biological activities (Capdevila et al. 1992). For instance, 12(R)-HETE formed by microsomal P450 by allyl oxidation of 20:4(n-6) is active as an inhibitor of Na+/K+...
ATPase (Fitzpatrick and Murphy 1989). The 18-20-hydroxyeicosatetraenoic acids formed have potent biological activities, including stimulation of rat renal cortex microsomal Na⁺/K⁺ ATPase by 19(S)-HETE (Escalante et al. 1990) and vasooactivity by 20-HETE (Escalante et al. 1990). Finally, epoxidation of 12:4(n-6) to form EETs has biological activity such as 14,15-epoxygenase activity in vitro (Fitzpatrick and Murphy 1989). What needs to be done now is to measure these 20:4(n-6) metabolites in the microsomes from rats fed ethanol to see whether any are increased in the in vivo steady state.

LITERATURE CITED


