Effect of Dietary Epigallocatechin-3-gallate on Cytochrome P450 2E1-Dependent Alcoholic Liver Damage: Enhancement of Fatty Acid Oxidation

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This study was designed to determine whether dietary epigallocatechin-3-gallate (EGCG), the most abundant catechin polyphenol in green tea, can protect the liver from cytochrome P450 2E1 (CYP2E1)-dependent alcoholic liver damage. Compared with an ethanol group, when EGCG was present in the ethanol diet, the formation of a fatty liver was significantly reduced and the serum aspartate transaminase (AST) and alanine transaminase (ALT) levels were much lower. Ethanol treatment significantly elevated hepatic CYP2E1 expression while simultaneously reducing hepatic phospho-acetyl CoA carboxylase (p-ACC) and carnitine palmitoyl-transferase 1 (CPT-1) levels. While EGCG markedly reversed the effect of ethanol on hepatic p-ACC and CPT-1 levels, it had no effect on the ethanol-induced elevation in CYP2E1 expression. EGCG prevents ethanol-induced hepatotoxicity and inhibits the development of a fatty liver. These effects were associated with improvements in p-ACC and CPT-1 levels. The use of EGCG might be useful in treating patients with an alcoholic fatty liver.

Key words: epigallocatechin-3-gallate (EGCG); alcohol; cytochrome P450 2E1 (CYP2E1); carnitine palmitoyl-transferase 1 (CPT-1); phospho-acetyl CoA carboxylase (p-ACC)

Considerable experimental and clinical evidence supports the notion that oxidative stress plays a key role in the liver injury caused by excessive alcohol consumption. For example, the degree of oxidative damage to the liver correlates positively with the amount of ethanol consumed. Moreover, ethanol metabolism generates large amounts of reactive oxygen species (ROS), which are known to cause liver pathology, in particular in alcoholic and toxic liver diseases.

CYP2E1 metabolizes many endogenous and exogenous small molecules such as acetone, glycerol, ethanol, acetaminophen, carbon tetrachloride, halothane, and nitrosamines. In other words, increased CYP2E1 expression, which is induced by excessive alcohol consumption, leads to increased oxygen radical production, thereby causing lipid peroxidation.

The increased hepatic expression of CYP2E1 in obese, diabetic, and alcoholic patients is believed to play a significant role in the pathogenesis of non-alcoholic steatohepatitis and alcoholic liver disease.

It has been proposed that natural dietary antioxidants might be protective in situations where ROS formation is excessive, such as in the hepatopaties induced by oxidative stress. One such dietary antioxidant is green tea, a rich source of polyphenols, especially flavanols and flavonols. Catechins are the predominant form of flavanols, and consist mainly of EGCG, epigallocatechin (EGC), epicatechin gallate (ECG), and epicatechin (EC). Recently, many of the beneficial effects of green tea are attributed to EGCG, its most abundant catechin.

This study was designed to evaluate the possible effect of dietary EGCG on the development of CYP2E1-dependent alcoholic liver damage in rats. Since ethanol impairs fat oxidation and stimulates lipogenesis, thereby promoting the development of a fatty liver, we also analyzed the expression of p-ACC and CPT-1, which are important regulators of hepatic fat metabolism known to be downregulated during the development of an alcoholic fatty liver.
Materials and Methods

**Chemicals.** EGCG (Teavigo) was obtained from DSM Nutritional Products (Basel, Switzerland). Teavigo is a highly purified extract of green tea leaves (*Camellia sinensis*) that contains > 94% EGCG and < 5% other catechins (< 3% GCG). A polyclonal sheep anti-human and rat CYP2E1 antibody was obtained from Chemicon International (Temecula, CA). An anti-pACC (Ser 79) antibody was from Cell Signaling Technology (Danvers, MA), and a rabbit anti-mouse CPT1-L antibody was from Alpha Diagnostic International (San Antonio, TX). A goat polyclonal IgG anti-beta-actin (C-11) antibody and a donkey anti-sheep IgG horseradish peroxidase-linked antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-mouse and anti-rabbit IgG linked antibody were from Santa Cruz Biotechnology and a donkey anti-sheep IgG horseradish peroxidase-linked antibody was obtained from Zymed (San Francisco, CA).

**Diet formulation.** Nutritionally adequate liquid diets formulated according to the method of Lieber and DeCarli were purchased from Dyets (Bethlehem, PA). The ethanol-containing diet consisted of 18% of total energy as protein, 35% as fat, 11% as carbohydrate, and 36% as ethanol. All control rats were pair-fed the same diet as the ethanol-fed rats, except that ethanol was replaced isonenergetically with carbohydrate. Thus both ethanol-fed and control rats ingested identical amounts of all nutrients, except for carbohydrates.

**Animals and treatments.** Male Wistar rats were purchased at 5 weeks of age from SLC Japan (Hamamatsu), and were used in all experiments. Both the animal care and study protocol employed were in accordance with Institutional Animal Care and Use Committee (IACUC) and Organization for Economic Cooperation and Development (OECD) guidelines. The rats were housed in individual cages at a temperature (23 ± 3 °C)- and relative humidity (40–60%)-controlled room. Lighting was adjusted automatically to yield a cycle of 12 h light and 12 h dark. Throughout the study, animals had *ad libitum* access to tab water. The local water supply authority regularly conducts water analyses to determine bacteriological and chemical contamination. During the study period, water contaminants were not present at levels that might interfere with the objective of this study. The rats were divided into four groups. The control group was fed for 7 weeks with the Lieber-DeCarli liquid diet (n = 6) and the EGCG group was fed for 7 weeks with the Lieber-DeCarli liquid diet supplemented with EGCG (3 g/l) (n = 4). The ethanol group was fed for 2 weeks with the control liquid Lieber-DeCarli diet, and then for 5 weeks with the ethanol-containing Lieber-DeCarli liquid diet (n = 10). The ethanol + EGCG group was fed for 2 weeks with the control Lieber-DeCarli liquid diet supplemented with EGCG (3 g/l), and then for 5 weeks with the ethanol Lieber-DeCarli liquid diet, which also contained EGCG (3 g/l, n = 10).

**Biochemical indicators of liver function.** At the end of the experimental term, all rats were weighed and blood was collected via the postcaval vein from anesthetized animals into a vacutainer serum-separating tube (BD Diagnostic, Sparks, MD). The serum was collected by centrifugation at 3,000 rpm for 10 min and then stored at −70 °C. The AST and ALT levels in the serum were analyzed with an automatic blood chemistry analyzer (Selectra E, Vital Scientific, Dieren, Netherlands).

**Histopathology.** Liver damage was determined by histological analysis. For this, the livers were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned into 3–4 um slices, stained with Hematoxylin and Eosin (H&E), and examined under a light microscope.

**Western blotting analyses.** The livers were perfused with ice-cold saline, and then immediately collected, weighed, frozen in liquid nitrogen, placed in 1.5-ml Eppendorf tubes, and stored at −70 °C until analysis. Fifty micrograms of total protein were analyzed under reducing conditions on 8% sodium dodecyl sulfate/polyacrylamide gels and blotted onto polyvinyl difluoride (PVDF) membranes. The blots were then blocked with 5% non-fat milk in TBS-T (150 mM NaCl, 50 mM Tris–HCl, 0.1% Tween-20, pH 7.4) for 3 h. After blocking, antibodies specific for CYP2E1, p-ACC, CPT-1, and beta-actin were diluted in blocking buffer and incubated with the membranes overnight in cold room with shaking. The membranes were then washed with TBS-T and incubated with secondary horseradish peroxidase-conjugated antibodies. The reaction products were detected with the ECL kit (Amersham Biosciences) according to the manufacturer’s instructions.

**Statistical analysis.** All data were expressed as means ± S.D. and statistical analyses were performed using Minitab® 14 software. All data were analyzed by one-way analysis of variance (ANOVA) followed by post hoc Tukey’s multiple range tests to determine treatment effect and to compare differences between group means. Differences were considered to be significant at $P < 0.05$.

**Results**

**Growth and liver weight**

Four groups of 5-week-old male Wistar rats were fed a nutritionally adequate liquid diet for 7 weeks. The control group (n = 6) received this diet alone, and the EGCG group (n = 4) received it supplemented with EGCG. The ethanol group (n = 10) received this diet for 2 weeks, and then were given the diet supplemented...
Enhancement of Fatty Acid Oxidation by EGCG

Fig. 1. Effects of Diet on the Body Weights of Wistar Rats.
Con, normal control group; EGCG, EGCG control group; EtOH, alcohol control group; EtOH + EGCG, alcohol-EGCG supplementation. The data are presented as means ± SD. The means were compared by one-way ANOVA. *P < 0.05 vs. control group. **P < 0.05 vs. ethanol group. EtOH, ethanol; EGCG, epigallocatechin-3-gallate.

Table 1. Effect of Diet on Wistar Rat Body and Liver Weights

<table>
<thead>
<tr>
<th>Group</th>
<th>Total body weight gain (g)</th>
<th>Liver (% of body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con</td>
<td>152.83 ± 9.66</td>
<td>2.42 ± 0.12</td>
</tr>
<tr>
<td>EGCG</td>
<td>151.69 ± 8.47</td>
<td>2.51 ± 0.41</td>
</tr>
<tr>
<td>EtOH</td>
<td>130.17 ± 15.06</td>
<td>2.55 ± 0.22</td>
</tr>
<tr>
<td>EtOH + EGCG</td>
<td>149.16 ± 18.17</td>
<td>2.60 ± 0.18</td>
</tr>
</tbody>
</table>

Con, normal control group; EGCG, EGCG control group; EtOH, alcohol control group; EtOH + EGCG, alcohol-EGCG supplementation. The means were compared by one-way ANOVA.

with alcohol for 5 weeks. The ethanol + EGCG group (n = 10) received the diet + EGCG for 2 weeks, and then diet + EGCG + ethanol for 5 weeks. The changes in body weight for the four groups during the experimental period are shown in Fig. 1. All animals gained weight over the period of the study and no difference in the final average bodyweights of the four groups was detected, although the ethanol group tended to have a lower body weight. The relative liver weights of the four groups remained unchanged (Table 1).

Biochemical indicators of liver function
Ethanol administration affected liver function in that it significantly elevated the levels of ALT and AST in serum, to 50.87 ± 15.26 and 99.98 ± 8.03 respectively (Table 2). However, when EGCG was provided at the same time as ethanol, there was a significant decrease in serum levels of ALT and AST, to 31.76 ± 6.86 and 63.60 ± 6.97 respectively.

Histological assessment of liver tissue
Histological examination of liver tissue sections (Fig. 2) revealed that the control group and EGCG group did not exhibit any abnormal appearances or histological changes, but ethanol administration caused severe fatty accumulation, inflammation, and necrosis. In contrast, when the ethanol-fed rats were simultaneously given EGCG, there was an obvious improvement in liver morphology.

Hepatic CYP2E1 expression
Figure 3 shows a representative western blot of CYP2E1 levels in the livers from the four rat groups. Ethanol treatment significantly increased the hepatic CYP2E1 levels relative to the levels observed in the controls rats (P < 0.05), but supplementation of the ethanol diet with EGCG did not significantly reduce these elevated hepatic CYP2E1 expression levels. Hence EGCG had no effect on the ethanol-induced over-expression of CYP2E1.

Hepatic p-ACC and CPT-1 expression
p-ACC and CPT-1 expression in the liver was also examined by western blot analysis. The ethanol group had significantly lower p-ACC and CPT-1 levels than

Table 2. Effect of EGCG and/or Ethanol on Serum ALT and AST Levels in Wistar Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>ALT (U/I)</th>
<th>AST (U/I)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con</td>
<td>18.80 ± 3.71</td>
<td>59.58 ± 3.82</td>
</tr>
<tr>
<td>EGCG</td>
<td>24.18 ± 7.52</td>
<td>65.93 ± 4.81</td>
</tr>
<tr>
<td>EtOH</td>
<td>50.87 ± 15.26*</td>
<td>99.98 ± 8.03*</td>
</tr>
<tr>
<td>EtOH + EGCG</td>
<td>31.76 ± 6.86**</td>
<td>63.60 ± 6.97**</td>
</tr>
</tbody>
</table>

Con, normal control group; EGCG, EGCG control group; EtOH, alcohol control group; EtOH + EGCG, alcohol-EGCG supplementation. The means were compared by one-way ANOVA. *P < 0.05 vs. control group. **P < 0.05 vs. ethanol group. EtOH, ethanol; EGCG, epigallocatechin-3-gallate.
the control group. Ethanol alone appeared to decrease p-ACC and CPT-1 immunoreactive protein expression in the liver, to 51.47% and 38.70% respectively, but EGCG significantly reversed this effect of ethanol on the hepatic p-ACC and CPT-1 levels, to 910.98% and 1,068.81% respectively, as compared with ethanol group (Fig. 4).

**Discussion**

This study was designed to evaluate the effect of dietary EGCG on the development of CYP2E1-dependent alcoholic liver damage. Since ethanol impairs fat oxidation, stimulates lipogenesis, and contributes to the development of a fatty liver,26 we also analyzed the hepatic expression of p-ACC and CPT-1, which regulate hepatic fat metabolism. These molecules are suppressed by ethanol, which promotes the development of alcoholic fatty liver. The liver is known to be the major organ responsible for the metabolism of drugs and toxic chemicals, and is therefore the primary organ that is affected by most toxic chemicals.28–30

It is known that chronic ethanol administration causes hepatotoxicity in rats. This is evidenced by morphological alterations in the liver that include signs of inflammation, necrosis, fibrosis, and fatty and collagen accumulation. These changes are also accompanied by substantial increases in the serum levels of transaminases, alkaline phosphatase (ALP), and r-glutamyltransferase (GGT). Furthermore, ethanol elevates the lipid peroxidation potential of the liver and decreases the activities of hepatic antioxidant enzymes that reflect oxidant stress in the liver.31 One mechanism by which ethanol has been proposed to mediate its toxic effects is through oxidative stress and oxidative damage due to increased production of ROS.32 Chronic excessive ethanol consumption increases hepatic CYP2E1 expression by several-fold and enhances its catalytic activity in hepatic microsomes by 20-fold.11 Induction of CYP2E1 is one of the main mechanisms by which ethanol increases ROS production and generates a state of oxidative stress in the liver.33,34

Consistently with the findings of other investigators,31,35,36 we found in this study that ethanol administration caused severe liver damage in rats, as evidenced by significant increases in serum AST and ALT levels and the presence of classic histopathological changes. We also observed that ethanol treatment significantly increased hepatic CYP2E1 expression relative to the levels observed in control rats ($P < 0.05$). These results are consistent with those of other studies, which suggest that ROS overproduction due to increased CYP2E1 expression can oxidatively damage liver DNA and contribute to hepatic injury.37

Xu et al. have suggested that ethanol exposure affects p-ACC and CPT-1 activity, and that this might help generate the alcoholic fatty liver.38 Indeed, we found that chronic ethanol intake significantly reduced hepatic p-ACC and CPT-1 levels. These molecules participate in the breakdown of fatty acids, as follows: Fatty acyl-
CoAs are broken down into shorter chains by a series of dehydrogenases, but long-chain fatty acids are unable to freely pass the mitochondrial membrane and their transfer across the outer mitochondrial membrane must be mediated by CPT-1. To do this, CPT-1 facilitates the coupling of carnitine to fatty acyl-CoA, thus generating an acylcarnitine that can be transferred across the outer membrane by CPT-1. The acylcarnitine then passes the inner mitochondrial membrane with the help of carnitine acylcarnitine translocase. Once inside the mitochondrion, the carnitine is removed from the acylchain by CPT-2. CPT-1 activity has been found to control the rate of this major step of hepatic oxidation. AMP-activated kinase (AMPK) has been found to increase the activity of a number of enzymes of lipid metabolism. For instance, activation of AMPK in liver has been found to phosphorylate and inactivate ACC. CPT-1 activity is regulated indirectly by ACC phosphorylation, as follows: ACC phosphorylation reduces the production of malonyl-CoA, an allosteric inhibitor of CPT-1. A decrease in malonyl-CoA has been found to promote CPT-1 activity and fatty acid oxidation in most tissues. P-ACC activity is down-regulated by ethanol because ethanol deactivates the ACC protein kinase (AMP-dependent protein kinase), either directly or by inducing TNF-α and adiponectin production. The downregulation of p-ACC then inhibits CPT-1 activity, thereby blocking fatty acid oxidation.

**Fig. 4. Western Blot Analysis of Hepatic p-ACC and CPT-1 Expression.**

Con, normal control group; EGCG, EGCG control group; EtOH, alcohol control group; EtOH + EGCG, alcohol-EGCG supplementation. A, Representative photos of western blot analyses performed using liver RNA samples from the control, EGCG, ethanol, and ethanol + EGCG groups. B, C, Ratios of hepatic p-ACC/beta-actin (B) and CPT-1/beta-actin (C) expression in the four experimental rat groups. *P < 0.05 vs. control group. **P < 0.05 vs. ethanol group. N = 3, the number of independent experiments. EtOH, ethanol; EGCG, epigallocatechin-3-gallate.
oxidation, elevating accumulation of fat in the liver, and thus promoting the development of a fatty liver.\(^{26}\)

Green tea contains relatively large amounts of polyphenols (mainly catechins and catechin derivatives), which have been reported to have antioxidant properties.\(^{42,48}\) It has been found to decrease the oxidative damage induced by cigarette smoking and alcohol intoxication; it also substantially decreased chemical carcinogen-induced tumorigenesis in an animal model.\(^{39}\) In particular, the major catechin in green tea, EGCG, has been found to prevent alcohol-induced liver injury.\(^{50}\) Moreover, Giakoustidis et al. reported that treatment with EGCG reduced the increment of serum levels of AST and ALT induced by liver injury. Our reports are in agreement with those of Giakoustidis et al.\(^{51}\) In the present study, we found that co-treatment of EGCG diminished ethanol-induced hepatic tissue injury, as shown by the improved liver architecture of the EGCG + ethanol rats as compared to the ethanol rats. There was also a corresponding reduction in the serum AST and ALT levels, which indicates that dietary EGCG protects the liver from ethanol toxicity.

One possible mechanism by which EGCG prevents ethanol-induced hepatotoxicity is inhibition of the catalytic activity of CYP2E1, similarly to theanine (the predominant amino acid in green tea),\(^{52}\) but Jimenez-Lopez et al. found that EGCG does not affect CYP2E1-mediated ethanol metabolism in the human hepatoma HepG2 cell line, which constitutively expresses human CYP2E1.\(^{53}\) Similarly, we found in this study that dietary EGCG had no effect on CYP2E1 expression in ethanol-administered rats. Thus the hepatoprotective activity of EGCG does not involve inhibition of CYP2E1 activity.

Another possibility is that EGCG prevents damaging lipid peroxidation chain reactions, since it is one of the catechol-type polyphenols, which are known to be potent free-radical scavengers.\(^{48,54,55}\) Indeed, it has been suggested that drugs such as EGCG that have antioxidant capacities and radical scavenging properties might be useful in treating alcohol-induced liver damage.\(^{50,56,57}\) However, in contrast to its antioxidative activity, some experiments in vitro indicate that EGCG produces reactive oxygen species. For example, EGCG promotes apoptosis and has bactericidal activity, which is attributed to its ability to reduce \(\text{O}_2^-\) to yield \(\text{H}_2\text{O}_2\).\(^{58,59}\) Maeta et al. have reported that tea polyphenols are able to act as prooxidants to cause a response to oxidative stress in yeast.\(^{60}\) Although there are reports indicating that EGCG induces oxidative stress by acting as a prooxidant, our study indicates that EGCG enhances the antioxidant capacity and prevents alcohol-induced liver injury in rats.

AMPK potently stimulates fatty-acid oxidation by inhibiting ACC activity.\(^{44,61}\) CPT-1 activity is regulated indirectly by ACC phosphorylation.\(^{26}\) CPT-1 activity has been found to control the rate of this major step in hepatic oxidation.\(^{39}\) This was associated with a significant reduction in the formation of an alcoholic fatty liver. We found that dietary EGCG significantly increased hepatic p-ACC and CPT-1 levels relative to those in the ethanol-alone group. Hwang et al. found that EGCG activates AMPK, a major kinase responsible for inactivation of ACC.\(^{52}\) These results are related with our results on p-ACC and CPT-1 in the pathway of metabolism of free fatty acids. Hence our findings suggest that EGCG increases the rate of hepatic fatty acid oxidation, thereby reducing alcoholic hepatic damage.

In conclusion, our results suggest that EGCG increases the rate of hepatic fatty acid oxidation and reduces alcoholic hepatic damage, but this effect does not appear to be mediated by the inhibition of CYP2E1. Instead, this effect of EGCG on fatty liver may be due in part to its antioxidant activity against ROS. Thus EGCG prevents ethanol-induced hepatotoxicity and might be useful in treating patients with an alcoholic fatty liver.

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