Kupffer cell-derived prostaglandin $E_2$ is involved in alcohol-induced fat accumulation in rat liver

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Enomoto, Nobuyuki, Kenichi Ikejima, Shunhei Yamashina, Ayako Enomoto, Teruhiro Nishitura, Tetsuro Nishimura, David A. Brenner, Peter Schemmer, Blair U. Bradford, Chantal A. Rivera, Zhi Zhong, and Ronald G. Thurman. Kupffer cell-derived prostaglandin $E_2$ is involved in alcohol-induced fat accumulation in rat liver. Am J Physiol Gastrointest Liver Physiol 279: G100–G106, 2000.—Destruction of Kupffer cells with gadolinium chloride ($GdCl_3$) and intestinal sterilization with antibiotics diminished ethanol-induced steatosis in the enteral ethanol feeding model. However, mechanisms of ethanol-induced fatty liver remain unclear. Accordingly, the role of Kupffer cells in ethanol-induced fat accumulation was studied. Rats were given ethanol (5 g/kg body wt) intragastrically, and tissue triglycerides were measured enzymatically. Kupffer cells were isolated 0–24 h after ethanol, and PGE$_2$ production was measured by ELISA, whereas inducible cyclooxygenase (COX-2) mRNA was detected by RT-PCR. As expected, ethanol increased liver triglycerides about threefold. This increase was blunted by antibiotics, $GdCl_3$, the dihydropyridine-type $Ca^{2+}$ channel blocker nimodipine, and the COX inhibitor indomethacin. Ethanol also increased PGE$_2$ production by Kupffer cells about threefold. This increase was also blunted significantly by antibiotics, nimodipine, and indomethacin. Furthermore, tissue triglycerides were increased about threefold by PGE$_2$ treatment in vivo as well as by a PGE$_2$, EP$_1$/EP$_2$ receptor agonist, whereas an EP$_1$/EP$_2$ agonist had no effect. Moreover, permeable cAMP analogs also increased triglyceride content in the liver significantly. We conclude that PGE$_2$ derived from Kupffer cells, which are activated by ethanol, interacts with prostanoid receptors on hepatocytes to increase cAMP, which causes triglyceride accumulation in the liver. This mechanism is one of many involved in fatty liver caused by ethanol.

Although alcohol is a well-known hepatotoxin, the mechanisms of pathology still remain unclear. Specifically, the role of lipid accumulation remains controversial. A single large dose of ethanol in rats causes a pronounced increase in liver triglycerides that is maximal in ~24 h and disappears after 48 h (4, 20). Although fatty liver clearly occurs, whether it is a causal event in ethanol-induced hepatitis and hepatic fibrosis still remains unclear.

Interestingly, inactivation of Kupffer cells with gadolinium chloride ($GdCl_3$) or decreasing gut-derived endotoxin by diminishing endotoxin by intestinal sterilization with antibiotics (polymyxin B and neomycin) decreased ethanol-induced steatosis in the enteral feeding model of Tsukamoto and French (1, 2). These treatments also prevented early ethanol-induced liver injury characterized by necrosis and focal inflammation. Moreover, similar phenomena were observed in rats treated with glycine, which inhibits Kupffer cells via activation of a glycine-gated chloride channel, and nimodipine, a dihydropyridine-type $Ca^{2+}$ channel blocker (15–18).

Therefore, it was hypothesized that activated Kupffer cells are somehow involved in mechanisms of ethanol-induced fatty liver. Accordingly, the aim of this study was to attempt to understand if Kupffer cells are indeed involved in hepatic fat accumulation, and if so, how.

MATERIALS AND METHODS

Animals and treatments. Female Sprague-Dawley rats weighing between 200 and 250 g were used in this study. All animals were given humane care in compliance with institutional guidelines. Rats were given ethanol (5 g/kg body wt po) before experiments (33, 34). Twenty-four rats were treated for 4 days with polymyxin B and neomycin (32) to prevent growth of intestinal bacteria, the main source of endotoxin in the gastrointestinal tract. On the basis of the results of preliminary experiments (2), polymyxin B (150 mg · kg$^{-1}$ · day$^{-1}$) and neomycin (450 mg · kg$^{-1}$ · day$^{-1}$) were given...
orally to achieve gut sterilization. Twenty-four rats were also treated with GdCl₃, a selective Kupffer cell toxicant, to inactivate Kupffer cells. In this experiment, a single dose of GdCl₃ (10 mg/kg) dissolved in acidic saline was administered intravenously to rats 24 h before ethanol treatment.

**Analytical methods.** Rats were forced to breathe into a closed heated chamber (37°C) for 20 s, and 1 ml of breath was collected using a gas-tight syringe to measure ethanol by gas chromatography. Blood ethanol concentration was assessed from breath ethanol (33, 34). Blood was collected from the portal vein in pyrogen-free heparinized syringes and centrifuged, and the plasma was stored at −20°C in pyrogen-free glass test tubes until endotoxin was measured using the Limulus amebocyte lysate assay (BioWhittaker, Walkersville, MD). Livers were formalin fixed, embedded in paraffin, and stained with hematoxylin and eosin to assess steatosis (24). Pathology was assessed in a blinded manner by one of the authors and by an independent pathologist with expertise in rodent liver.

**Assay for hepatic triglycerides.** To assess triglyceride content, liver tissue was homogenized in an equal volume of normal saline and extracted with a mixture of chloroform and methanol (2:1) as described previously (4, 6, 13). Zeolite was added to remove phospholipids. The resulting extract was dried under nitrogen and dissolved in Plasmanate (1 ml), and triglycerides were measured enzymatically (4–6).

**Kupffer cell preparation and culture.** Kupffer cells were isolated by collagenase digestion and differential centrifugation using Percoll (Pharmacia, Uppsala, Sweden) as described previously (27) with slight modifications. Briefly, the liver was perfused through the portal vein with Ca²⁺- and Mg²⁺-free Hanks’ balanced salt solution (HBSS) at 37°C for 5 min at a flow rate of 26 ml/min. Subsequent perfusion was with HBSS containing 0.025% collagenase IV (Sigma Chemical, St. Louis, MO) at 37°C for 5 min. After the liver was digested, it was excited and cut into small pieces in collagenase buffer. The suspension was filtered through nylon gauze mesh, and the filtrate was centrifuged at 450 g for 10 min at 4°C. Cell pellets were resuspended in buffer, parenchymal cells were removed by centrifugation at 50 g for 3 min, and the nonparenchymal cell fraction was washed twice with buffer. Cells were centrifuged on a density cushion of Percoll at 1,000 g for 15 min, and the Kupffer cell fraction was collected and washed with buffer again. Viability of cells determined by trypan blue exclusion was >90%. Cells were seeded onto 24-well culture plates and cultured in RPMI 1640 (GIBCO Laboratories Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum and 10 mMol/l HEPES and antibiotics (100 µU/ml penicillin G and 100 µg/ml streptomycin sulfate) at 37°C with 5% CO₂. Non-adherent cells were removed after 15 min by replacing buffer, and cells were cultured for 4 h before experiments.

**Measurement of PGE₂ in conditioned media from cultured Kupffer cells.** Kupffer cells isolated from rats were kept in primary culture for 4 h, and supernatants were analyzed for PGE₂ by competitive RIA using ¹²⁵I-labeled PGE₂ from Advanced Magnetics (Cambridge, MA). Although this antibody reacts with PGE₂, there is <2% cross-reactivity with other prostaglandins, arachidonic acid, and thromboxane.

**RNA preparation and RT-PCR for inducible cyclooxygenase mRNA.** Total RNA was prepared by guanidium/CSCl centrifugation as described previously (7, 23, 25, 26). The integrity and concentration of RNA was determined by measuring absorbance at 260 nm followed by electrophoresis on agarose gels. First-strand cDNA was transcribed from 1 µg RNA using Moloney murine leukemia virus RT (Life Technologies, Gaithersburg, MD) and an oligo(dT)₁₆ primer (Perkin Elmer), and PCR was performed using a GeneAmp PCR system 9600 (Perkin Elmer, Foster City, CA). The primer sets used in this study are shown in Table 1 (12). We amplified 1 µl of cDNA in a 50 µl reaction buffer containing 10 pmol of forward and reverse primers, 2.5 U Taq DNA polymerase, 250 mM 2′-deoxynucleoside 5′-triphosphates (dNTPs), and 1× PCR buffer (Perkin Elmer). The reaction mixture without enzyme and dNTPs was heated at 100°C for 4 min, then a mixture of Taq polymerase and dNTP was added at 80°C. Thereafter, 40 cycles of denaturing at 94°C for 60 s, annealing at 50°C for 90 s, and extension at 72°C for 120 s followed by final extension at 72°C for 10 min were carried out. The size of the PCR products was verified by electrophoresis in 2% agarose gels followed by ethidium bromide staining. Densitometric analysis using NIH image software was performed for semiquantification of PCR products.

**Tissue extraction.** One part of the liver was homogenized with 9 parts 10% TCA using a Polytron homogenizer. The supernatant was centrifuged with 5 vol of water-saturated ether. The ether layer then was removed, and extraction of the aqueous layer was repeated two times. Residual ether was removed from the aqueous layer.

**Measurement of CAMP.** Intracellular CAMP was measured in suspensions of parenchymal cells by RIA using ¹²⁵I-labeled-cAMP from Biomedical Technologies (31). Parenchymal cells were incubated in RPMI 1640 medium containing various concentrations of PGE₂ at 37°C. For some experiments, 0.5 mM IBMX was preincubated with parenchymal cells for 2 min before the addition of PGE₂. After 5 min, cells were washed with cold PBS, centrifuged in polypropylene tubes, and treated with 0.05 M HCl. Tubes were then placed in boiling water for 3 min. Standards and unknowns were combined with tracer solution and antibody and were incubated 18–20 h at 4°C. Acetate buffer (1 ml) was added, the tubes were centrifuged, and the pellets were separated from the supernatant. Radioactivity in the precipitate was counted and compared with known values from a standard curve.

**Statistical analysis.** All results were expressed as means ± SE. Statistical differences between means were determined using ANOVA or ANOVA on ranks as appropriate. P < 0.05 was selected before the study to reflect significance.

**RESULTS**

**Effect of antibiotics.** After oral administration of ethanol (5 g/kg) to untreated normal rats, blood ethanol

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Oligonucleotide Primers</th>
<th>Size of PCR Product, bp</th>
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<tbody>
<tr>
<td>COX-2</td>
<td>Forward: 5′-ACT TGC TCA CTT TGT TGA GTC ATT C-3′</td>
<td>583</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5′-TTT GAT TAG TAC TGT AGG GTT AAT G-3′</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: 5′-CAT CTC TGC CCC CTC TGC TGA-3′</td>
<td>306</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5′-GGA TGA CCT TGC CCA CAG CCT-3′</td>
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COX-2, inducible cyclooxygenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
levels increased gradually and reached ~250 mg/dl after 90 min (Table 2). Similar results were obtained in rats treated with antibiotics. Plasma endotoxin levels in the portal vein were increased about fourfold to ~90 pg/ml 1.5 h after ethanol (Table 2). This effect was blocked by antibiotics.

Effect of drugs affecting Kupffer cells on hepatic triglyceride levels and liver histology. Liver weight was measured before experiments, and no significant differences between the groups studied were detected (data not shown). Liver specimens were collected for histology 24 h after administration of ethanol (5g/kg

Table 2. Effect of acute ethanol and antibiotics on blood alcohol and endotoxin levels

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Alcohol, mg/dl</th>
<th>Endotoxin, pg/ml</th>
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</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>268 ± 38</td>
<td>88 ± 7</td>
</tr>
<tr>
<td>Antibiotics + ethanol</td>
<td>245 ± 34</td>
<td>23 ± 1*</td>
</tr>
</tbody>
</table>

Values are means ± SE of 4 individual rats or samples. Rats were given ethanol, which was analyzed in breath by gas chromatography at 90 min as described in MATERIALS AND METHODS. Portal blood endotoxin was determined by the Limulus amebocyte lysate pyrogen test at 90 min. Controls without ethanol were below the detection limits in the presence and absence of antibiotics. *P < 0.05 vs. ethanol by Student’s t-test.
Histology was normal in control rats (Fig. 1A), whereas ethanol caused steatosis in the liver as expected (Fig. 1B). Histological changes were blunted almost completely by intestinal sterilization with antibiotics, inactivation of Kupffer cells with GdCl₃, inhibition of intracellular Ca²⁺ concentration influx with the Ca²⁺ channel blocker nimodipine, and the cyclooxigenase (COX) inhibitor indomethacin (Fig. 1, C–F).

Mean liver triglycerides in vehicle-treated controls were 5.5 ± 1.0 mg/g liver (Fig. 2), and values were increased about threefold by ethanol as expected. This increase was prevented nearly completely by prior treatment of rats with antibiotics, GdCl₃, nimodipine, or indomethacin.

**PGE₂ production by conditioned media from Kupffer cells treated with ethanol.** PGE₂ production by Kupffer cells from untreated control rats was 43 ± 5 pmol·10⁶ cells⁻¹·4 h⁻¹ (Fig. 3). Ethanol treatment increased values about threefold. This increase was also blunted significantly by antibiotics, nimodipine, or indomethacin.

**Effect of PGE₂, ethanol, and cAMP analogs on tissue triglycerides.** Liver specimens were collected for histology and measurement of tissue triglycerides 24 h after injection of PGE₂ (1 mg/kg iv). Tissue triglycerides were elevated nearly twofold by PGE₂ or ethanol treatment (Fig. 4). Interestingly, ethanol and PGE₂ were additive under these conditions.

Analogs of PGE₂, 17-phenyl-omega-trinor-PGE₂ (17-PGE₂, an EP₁/EP₃ agonist) or 11-deoxy PGE₁ (11-PGE₁, an EP₂/EP₄ agonist), were injected (1 mg/kg iv) 24 h before tissue triglyceride measurements. Cell permeable cAMP analogs were also examined for their effect on tissue triglycerides. Dibutryl cAMP (DB-cAMP; 1 mg/kg) and 8-bromoadenosine cAMP (8-BrcAMP; 1 mg/kg) were injected intravenously 8 h before tissue triglyceride measurements. The level of
cAMP in the liver was increased significantly by these treatments (Table 3). Moreover, tissue triglycerides were elevated about threefold by an EP2/EP4 agonist, but an EP1/EP3 agonist had no effect (Fig. 5). CAMP analogs also increased triglycerides two- to threefold.

**TABLE 3. Effect of cAMP analogs on liver levels of cAMP**

<table>
<thead>
<tr>
<th></th>
<th>cAMP, pmol/g</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>629 ± 36</td>
</tr>
<tr>
<td>8-BrcAMP</td>
<td>880 ± 101a</td>
</tr>
<tr>
<td>DBcAMP</td>
<td>1,157 ± 150a</td>
</tr>
</tbody>
</table>

Values are means ± SE of 4 samples from individual rats. Rats were given cAMP analogs, and the liver level of cAMP was measured by RIA 8 h after administration as described in MATERIALS AND METHODS. 8-BrcAMP, 8-bromoadenosine cAMP; DBcAMP, dibutyryl cAMP. *P < 0.05 vs. control by ANOVA and Bonferroni’s post-hoc test.

cAMP in the liver was increased significantly by these treatments (Table 3). Moreover, tissue triglycerides were elevated about threefold by an EP2/EP4 agonist, but an EP1/EP3 agonist had no effect (Fig. 5). CAMP analogs also increased triglycerides two- to threefold.

**Effect of drugs affecting Kupffer cells on expression of inducible COX mRNA from rat liver treated with ethanol.** Inducible COX (COX-2) mRNA expression was undetectable in livers from untreated control rats but was detected within 90 min after lipopolysaccharide (LPS; 1 mg/kg). Moreover, COX-2 mRNA expression was increased by treatment with ethanol (2 h) nearly as much as with LPS (Fig. 6). This increase was blocked totally by antibiotics, GdCl3, nimodipine, and indomethacin.

**DISCUSSION**

Kupffer cells and endotoxin are involved in mechanisms of fatty liver. Many physiological factors participate in ethanol-induced fatty liver (22). For example,

**Fig. 6. RT-PCR analysis of inducible COX (COX-2) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.**

A: total liver RNA isolated 2 h after ethanol (5 g/kg) administration was used to detect COX-2 mRNA. GAPDH was also detected as a housekeeping gene and aX174/Hae III was used to determine the size of PCR products. Lane 1, LPS-treated positive control; lane 2, untreated control; lane 3, ethanol; lane 4, antibiotics + ethanol; lane 5, GdCl3 + ethanol; lane 6, nimodipine + ethanol; lane 7, indomethacin + ethanol; lane 8, no RNA. Gel is representative of 4 individual experiments. MW, molecular weight. B: results are means ± SE; n = 4. *P < 0.05 vs. control. #P < 0.05 vs. ethanol by ANOVA and Bonferroni’s post hoc test.

Fatty acid synthesis increases, fatty acid oxidation decreases, release of lipoproteins diminishes, and systemic adrenergic activity increases cause peripheral lipolysis. With this study, a role for Kupffer cells can be added to this list. One possible explanation for the results observed here is as follows. A single large dose of ethanol increases gut-derived endotoxin in the circulation (Table 2). Endotoxin is removed from the circulation primarily by Kupffer cells, which are activated leading to rapid increases in intracellular Ca2+, which in turn activates phospholipase A2. This increases...
PGE$_2$ synthesis via mechanisms involving COX-2 (21) (Fig. 6). PGE$_2$ then acts on receptors on hepatocytes to increase triglycerides in the liver (Fig. 7). Previous studies (1, 2) showed that inactivation of Kupffer cells with GdCl$_3$ and intestinal sterilization with antibiotics (polymyxin B and neomycin) prevented alcohol-induced steatosis in the enteral feeding model of Tsukamoto and French. However, how the Kupffer cell is involved in mechanisms of hepatic triglyceride accumulation remains unclear. Here, one single large dose of ethanol also increased neutral lipid in the liver (4, 20) (Figs. 1 and 2). Moreover, in this study, inactivation of Kupffer cells with GdCl$_3$ intestinal sterilization with antibiotics, prevention of influx of extracellular Ca$^{2+}$ with a Ca$^{2+}$ channel blocker, and inhibition of COX all reduced hepatic lipid accumulation (Figs. 1 and 2). Accordingly, it is concluded that Kupffer cells and endotoxin are involved in mechanisms of fatty liver.

**PGE$_2$ is involved in triglyceride accumulation in hepatocytes.** Eicosanoids are bioactive lipids produced in large quantities by macrophages from arachidonic acid that is released from membrane lipids through the action of phospholipase A$_2$ (3, 9). Arachidonic acid produced via the COX pathway leads to the formation of prostaglandins, key mediators of cell signaling between Kupffer cells and hepatocytes (28). PGE$_2$ production from Kupffer cells was enhanced by ethanol, a phenomenon blunted by antibiotics, nimodipine, and indomethacin (Fig. 3). Triglyceride accumulation was also increased by PGE$_2$, and this effect was enhanced by the addition of ethanol (Fig. 4). Many of the known biological effects of PGE$_2$ are mediated through interaction of PGE$_2$ with specific receptors (8, 14), and at least four subtypes (EP$_1$, EP$_2$, EP$_3$, and EP$_4$) have been characterized pharmacologically and cloned from at least one species (8, 19). The specific receptor subtypes are known to be coupled to different signal transduction pathways. EP$_1$ receptors are coupled to inositol phospholipid turnover, resulting in an increase of intracellular Ca$^{2+}$ concentration. EP$_2$/EP$_4$ receptors act via G$_{	ext{i/o}}$ proteins and increase cAMP, whereas EP$_3$ receptors are coupled to G$_{	ext{i/o}}$ and decrease cAMP (28). In this study, 11-deoxy PGE$_1$, an EP$_2$/EP$_4$ agonist, enhanced triglyceride production, whereas 17-PGE$_2$, an EP$_1$/EP$_3$ agonist, had no effect on tissue triglyceride levels (Fig. 5). Moreover, cAMP increased triglyceride accumulation (Fig. 5). Accordingly, it is concluded that Kupffer cell-derived PGE$_2$ is involved in triglyceride accumulation in hepatocytes via mechanisms dependent on hepatocyte EP$_2$/EP$_4$ receptors and cAMP. Indeed, it has been reported that ethanol increases cAMP, and it is well known that PGE$_2$ increases cAMP (20).

**PGE$_2$ is regulated by COX-2.** PGE$_2$ is synthesized in Kupffer cells via the COX pathway (29, 30), and the COX-2 gene may play an important role in liver injury. Dinchuk et al. (10) showed that COX-2 mediates endotoxin-induced liver injury in experiments with COX-2-deficient mice. On the other hand, indomethacin, a nonspecific COX inhibitor, prevented histological changes in the liver and PGE$_2$ production from Kupffer cells caused by gut-derived endotoxin (Figs. 1–3). Recently, Nanji et al. (25) showed that upregulation of COX-2 in chronic alcoholic liver injury increased synthesis of inflammatory and vasoactive eicosanoids. Furthermore, Nanji et al. (26) showed that dietary saturated fatty acids suppressed COX-2 expression in alcohol-induced liver injury. In this study, COX-2 mRNA expression was increased by treatment with ethanol in only 2 h (Fig. 6). This expression was totally blocked by treatment with antibiotics (Fig. 6). Therefore, these data support the hypothesis that endotoxin-induced increases in COX-2 expression increase PGE$_2$ production.

In summary, one large dose of ethanol is sufficient to increase gut-derived endotoxin in the circulation. Endotoxin is removed primarily by Kupffer cells that are activated, leading to rapid increases in COX-2 and intracellular Ca$^{2+}$, the latter of which in turn activates...
phospholipase A2. This increases PGE₂, which acts on receptors in hepatocytes to increase accumulation of triglycerides. This pathway is one of many physiological processes involved in mechanisms of fatty liver caused by ethanol (Fig. 7).

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Portions of this work have been presented previously in abstract form (see Ref. 11).

REFERENCES