Medium-chain triglycerides inhibit free radical formation and TNF-α production in rats given enteral ethanol

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Kono, Hiroshi, Nobuyuki Enomoto, Henry D. Connor, Michael D. Wheeler, Blair U. Bradford, Chantal A. Rivera, Maria B. Kadiiska, Ronald P. Mason, and Ronald G. Thurman. Medium-chain triglycerides inhibit free radical formation and TNF-α production in rats given enteral ethanol. Am. J. Physiol. Gastrointest. Liver Physiol. 278: G467–G476, 2000.—This study determined whether free radical formation by the liver, tumor necrosis factor (TNF-α), production by isolated Kupffer cells, and plasma endotoxin are affected by dietary saturated fat. Rats were fed enteral ethanol and corn oil (E-CO) or medium-chain triglycerides (E-MCT) and control rats received corn oil (C-CO) or medium-chain triglycerides (C-MCT) for 2 wk. E-CO rats developed moderate fatty infiltration and slight inflammation; however, E-MCT prevented liver injury. Serum aspartate aminotransferase levels, gut permeability, and plasma endotoxin doubled with E-CO but were blunted ~50% with E-MCT. In Kupffer cells from E-CO rats, intracellular calcium was elevated by lipopolysaccharide (LPS) in a dose-dependent manner. In cells from E-MCT rats, increases were blunted by ~40–50% at all concentrations of LPS. The LPS-induced increase in TNF-α production by Kupffer cells was dose dependent and was blunted by 40% by MCT. E-CO increased radical adducts and was reduced ~50% by MCT. MCT prevent early alcohol-induced liver injury, in part, by inhibition of free radical formation and TNF-α production by inhibition of endotoxin-mediated activation of Kupffer cells.

tumor necrosis factor-α; intracellular calcium; free radicals; alcohol

The establishment of a continuous intragastric in vivo enteral feeding protocol in the rat by Tsukamoto and French (17, 46) was a major development in alcohol research. With this model, not only is steatosis observed, which is characteristic of several animal models, but inflammation and necrosis also occur in ~2–4 wk and fibrosis begins to develop in 8–10 wk. Inactivation of Kupffer cells with GdCl3 prevented early alcohol-induced liver injury (1), diminished hypoxia (4), and prevented free radical formation (28). Furthermore, intestinal sterilization with antibiotics (2) or lactobacillus feeding (34) diminished endotoxin and minimized liver injury. Moreover, treatment with antibody to tumor necrosis factor (TNF-α) prevented early alcohol-induced liver injury in the Tsukamoto-French model (24). Alcohol-induced liver injury was also prevented in TNF receptor-1 knockout mice given enteral ethanol intragastrically (50). These results are consistent with the hypothesis that Kupffer cells activated by gut-derived endotoxin play an important role in the mechanism of alcohol-induced liver injury by producing TNF-α (43).

Activated Kupffer cells produce mediators, including inflammatory cytokines, eicosanoids, proteases, and oxygen radicals. Indeed, plasma levels of TNF-α (6), interleukin (IL)-1 (32), and IL-6 were increased in patients with severe alcoholic hepatitis, and values correlated with the clinical course of the disease (22). Calcium is essential for activation of Kupffer cells (26), which contain voltage-dependent Ca2⁺ channels (21), and they are easier to activate after chronic ethanol treatment (19). Moreover, ethanol causes both tolerance and sensitization of Kupffer cells (15). On the basis of sensitivity to antibiotics, it was concluded that both of these phenomena were caused by gut-derived endotoxin and that sensitization involves increases in the endotoxin receptor CD14 (15).

The type of dietary fat is important in the pathogenesis of alcoholic liver injury (35). It is known that saturated fat prevents early alcohol-induced liver injury (37); however, the mechanisms remain unclear. Medium-chain triglycerides (saturated fat) decreased TNF-α mRNA expression in the liver and prevented liver injury in the Tsukamoto-French model (38). Accordingly, the purpose of this study was to determine whether plasma endotoxin levels, TNF-α production by isolated Kupffer cells, and free radical formation by the liver were affected by dietary saturated fat. Preliminary accounts of this work have appeared elsewhere (29).

MATERIALS AND METHODS

Animals and diets. Male Wistar rats (275–300 g, n = 6) were fed enteral ethanol (10–14 g·kg⁻¹·day⁻¹) and a diet (190 kcal·kg body wt⁻¹·day⁻¹) containing either corn oil...
(unsaturated fat) or medium-chain triglycerides (saturated fat) without supplemental essential fatty acids continuously for up to 2 wk via intragastric feeding using the enteral protocol developed by Tsukamoto and French (17, 46). Control rats were fed a high-fat diet containing corn oil or medium-chain triglycerides without ethanol. A liquid diet described first by Thompson and Retz (42) and supplemented with lipotropes as described by Morimoto et al. was used (33). It contained corn oil or medium-chain triglycerides as fat (37% of total calories), protein (23%), carbohydrate (5%), minerals and vitamins, plus ethanol (35–40% of total calories) or isocaloric maltose-dextrin (control diet) as described elsewhere (45).

Clinical chemistry. Ethanol concentration in urine, which is representative of blood alcohol levels (5), was measured daily. Rats were housed in metabolic cages that separated urine from feces, and urine was collected over 24 h in bottles containing mineral oil to prevent evaporation. Each day at 9:00 AM, urine collection bottles were changed and a 1-ml sample was stored at −20°C in a microtube for later analysis. Ethanol concentration was determined by measuring absorbance at 366 nm resulting from the reduction of NADH to NAD by alcohol dehydrogenase (7).

Blood was collected via the tail vein once a week and centrifuged. Serum was stored at −20°C in a microtube until assayed for aspartate aminotransferase (AST) by standard enzymatic procedures (7).

Endotoxin assay. For measurement of plasma endotoxin, blood was collected via the portal vein in pyrogen-free heparinized syringes and centrifuged at 1,200 rpm for 10 min. Plasma was stored at −20°C in pyrogen-free glass tubes until measurement of endotoxin using a Limulus amoebocyte lysate test kit (Kinetic-QCL, BioWhittaker, Walkersville, MD; Ref. 31).

Pathological evaluation. After 2 wk of ethanol treatment, liver and gut sections from the small or large intestine near the cecum were formalin fixed, embedded in paraffin, and stained with hematoxylin-eosin to assess steatosis, inflammation, and necrosis. Liver pathology was scored as described by Nanji et al. (35): steatosis (percentage of liver cells containing fat): <25% = 1+, <50% = 2+, <75% = 3+, 75% = 4++; inflammation and necrosis: one focus per low-power field = 1+, two or more foci = 2+. Pathology was scored in a blinded manner by one of the authors and by an outside expert in rodent liver pathology.

Alcohol metabolism. Ethanol-containing diets were removed immediately before measurement. Rats were forced to breathe into a closed, heated chamber (37°C) for 20 s, and 1 ml of breath was collected with a gas-tight syringe. Concentration of ethanol in breath was determined by gas chromatography, and rates of alcohol metabolism were calculated from the rate of oxidation of pyrogallol as described elsewhere (12).

Kupffer cell preparation and culture. Ethanol was removed 24 h before isolation because Kupffer cells from rats treated with ethanol acutely for 24 h were sensitized to LPS (15). Kupffer cells were isolated by collagenase digestion and differential centrifugation using Percoll (Pharmacia, Uppsala, Sweden) as described elsewhere, with slight modifications (39). 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. Subsequently, the liver was perfused 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 excised and cut into small pieces and placed in collagenase buffer. The suspension was filtered through nylon gauze, 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 from trypan blue exclusion was >90%. Cells were seeded onto 25-mm glass coverslips and cultured in DMEM (GIBCO-Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES, and antibiotics (100 U/ml of penicillin G and 100 µg/ml of streptomycin sulfate) at 37°C with 5% CO2. Nonadherent cells were removed after 1 h by replacing the culture medium. All adherent cells phagocytosed latex beads, indicating that they were Kupffer cells. Cells were cultured 24 h before experiments.

Measurement of intracellular calcium. Intracellular calcium concentration ([Ca2+]i) was measured fluorometrically using the Ca2+ indicator dye fura 2 and a microspectrofluorometer (Photon Technology International, South Brunswick, NJ) interfaced with an inverted microscope (Diaphot, Nikon, Japan). Kupffer cells were incubated in modified Hanks’ buffer (in mM: 115 NaCl, 5 KCl, 0.3 Na2HPO4, 5.6 glucose, 0.8 MgSO4, 1.26 CaCl2, and 15 HEPES, pH 7.4), containing 5 µM fura 2-AM (Molecular Probes, Eugene, OR) and 0.03% Pluronic F127 (BASF Wyandotte, Wyandotte, MI) at room temperature for 60 min. Coverslips plated with Kupffer cells were rinsed and placed in chambers with buffer at room temperature. Changes in fluorescence intensity of fura 2 at excitation wavelengths of 340 and 380 nm and emission at 510 nm were monitored in individual Kupffer cells. Each value was corrected by subtracting the system dark noise and autofluorescence, assessed by quenching fura 2 fluorescence with Mn2+ as described previously (15). [Ca2+]i was determined from the equation

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\frac{[Ca^{2+}]_i}{R_0} = K_d (R - R_{min}) (R_{max} - R) (F_0/F_s)
\]

where F0/Fs is the ratio of fluorescence intensities evoked by 380-nm light from fura 2 pentapotassium salt loaded in cells using a buffer containing 3 mM EGTA and 1 µM ionomycin ([Ca2+]i min) or 10 mM Ca2+ and 1 µM ionomycin ([Ca2+]i max); R is the ratio of fluorescence intensities at excitation wavelengths of 340 and 380 nm; and Rmax and Rmin are values of R at [Ca2+]i max and [Ca2+]i min, respectively. The values of these constants were determined at the end of each experiment, and a dissociation constant (Kd) of 135 nM was used (20).

TNF-α production by Kupffer cells. Kupffer cells were seeded onto 24-well plates and cultured in DMEM supplemented with 10% FBS and antibiotics at 37°C in the presence of 5% CO2. Cells were incubated with fresh medium containing LPS (10–100 ng/ml supplemented with 5% rat serum) for an additional 4 h. Medium was collected and kept at −80°C until assay. TNF-α in the culture medium was mea-
sured using an ELISA kit (Genzyme, Cambridge, MA), and data were corrected for dilution.

Kupffer cell preparation. Kupffer cells were isolated by collagenase digestion and differential centrifugation using Percoll as described elsewhere, with slight modifications (39). Cells were plated on plastic culture dishes and cultured in RPMI 1640 medium (GIBCO-Life Technologies) supplemented with 25 mM HEPES, 10% FBS and antibiotics (100 U/ml of penicillin G and 100 µg/ml of streptomycin sulfate). After 1 h of incubation, Kupffer cells were scraped with a sterile cell scraper and pelleted by centrifugation at 500 g for 7 min. Cell pellets were resuspended in 250 µl of suspension buffer with Triton X-100, agitated for 15 min at 4°C, and centrifuged at 12,000 g for 10 min at 4°C. The supernatant was removed, and the pellet was resuspended in suspension buffer. Protein was stored at −20°C for subsequent Western blotting.

Western blotting for CD14. Extracted proteins (50 µg) were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes. Membranes were blocked by Tris-buffered saline-Tween 20 containing 5% skim milk and probed with mouse anti-rat ED9 monoclonal antibody (Sero-tech, Oxford, UK), followed by HRP-conjugated secondary antibody as appropriate. Membranes were incubated with a chemiluminescence substrate (ECL reagent, Amersham Life Science, Amersham, UK) and exposed to X-OMAT films (Eastman Kodak, Rochester, NY).

Collection of bile and free radical detection. Ethanol concentration in the breath was analyzed by gas chromatography to verify that levels were between 200 and 250 mg/dl when experiments were initiated (18). The rat was anesthetized with pentobarbital sodium (75 mg/kg), the abdomen was opened, and the spin trap α-(4-pyridyl-1-oxide)-N-tet-butyl nitronate (POBN, 1 g/kg) was administered intravenously. The proximal bile duct was cannulated with a small length of PE-10 tubing, and bile samples were collected at 30-min intervals for 3 h into 35 µl of 0.5 mM Desferal (deferoxamine mesylate) to prevent ex vivo radical formation. Samples were stored at −80°C until analysis of free radical adducts by electron spin resonance (ESR) spectroscopy (28). Samples were thawed and placed in a quartz flat cell, and ESR spectra were obtained using a Varian E-109 spectrometer equipped with a TM110 cavity. Instrument conditions were as follows: 20-mW microwave power, 1.0-G modulation amplitude, 80-G scan width, 16-min scan, and 1-s time constant. Data were collected with an IBM-type computer interfaced to a spectrometer. Simulations and double integrations of spectra to determine amplitude were carried out with a computer program (14).

Statistics. Data are expressed as mean values ± SE. ANOVA or Student’s t-test was used for determination of statistical significance as appropriate. For comparison of pathological scores, the Mann-Whitney rank-sum test was used.
A P value <0.05 was selected before the study as the level of significance.

RESULTS

Body weight. To allow for full recovery from surgery, diets were initiated 1 wk after surgery. No complications were observed by feeding medium-chain triglyceride for 2 wk. The mean body weight gain of rats infused with a high-fat control diet containing corn oil without ethanol was 5.2 ± 0.4 g/day, whereas rats receiving medium-chain triglycerides grew at a rate of 5.0 ± 0.2 g/day, as expected. The mean body weight gain of rats fed an ethanol-containing diet was 4.9 ± 0.4 (corn oil) and 4.6 ± 0.2 (medium-chain triglycerides) g/day. There were no significant differences in body weight gain between control and ethanol-treated groups.

Ethanol concentrations in urine and ethanol metabolism. Daily urine alcohol concentrations in rats fed ethanol with corn oil and medium-chain triglycerides are depicted in Fig. 1. As reported previously by several groups (1, 36, 44), alcohol levels fluctuate in a cyclic pattern from zero to >300 mg/dl for unknown reasons. Similar patterns were observed here in rats fed corn oil and medium-chain triglycerides. There were no significant differences in the cyclic pattern and mean urine alcohol concentrations between rats fed corn oil (135 ± 11 mg/dl) and medium-chain triglycerides (140 ± 11 mg/dl).

Fig. 3. Effect of chronic ethanol and lipid type on gut permeability. Gut permeability was measured as described in MATERIALS AND METHODS. Data represent means ± SE (n = 4 rats). HRP, horseradish peroxidase. *P < 0.05 compared with rats fed corn oil without ethanol and #P < 0.05 compared with rats fed corn oil with ethanol by ANOVA and Bonferroni's post hoc test.

Fig. 4. Effect of chronic ethanol and lipid type on gut histology. Gut sections from rats given high-fat control diet (A: corn oil; B: medium-chain triglycerides) and high-fat ethanol-containing diet (C: corn oil; D: medium-chain triglycerides) are shown. Representative photomicrographs are shown. Original magnification, ×200.
After 2 wk of ethanol, the rate of ethanol elimination was \(6.5 \pm 0.4 \text{ mmol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}\) in rats fed corn oil and \(6.4 \pm 0.7 \text{ mmol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}\) in rats fed medium-chain triglycerides. There were no significant differences between groups.

Serum transaminase levels and endotoxin. In control groups, serum AST levels were \(~45\) IU/l after 2 wk. Enteral ethanol with corn oil caused an approximately twofold increase over control (Fig. 2). In contrast, values were blunted significantly by \(~50\%\) by dietary medium-chain triglycerides.

Plasma endotoxin levels were <10 pg/ml in control groups after 2 wk. In rats fed corn oil with ethanol, however, values were increased significantly to \(89 \pm 20\) pg/ml \((n = 6)\). In contrast, this increase was blunted significantly >50% by dietary medium-chain triglycerides \((29 \pm 3\) pg/ml, \(P < 0.05)\).

Gut permeability and pathology. In control groups, gut permeability to HRP was \(~50\) U/l after 2 wk of the high-fat diet. Enteral ethanol with corn oil caused an approximately twofold increase over control values. This increase was significantly blunted to the same values as those for control dietary medium-chain triglycerides (Fig. 3).

No pathological changes were observed in gut sections from rats fed a high-fat control diet with corn oil or medium-chain triglycerides for 2 wk (Fig. 4, A and B). In contrast, destructive structure of the mucosal layer, hemorrhagic changes, and infiltrating inflammatory cells were observed in intestinal sections from rats fed ethanol with corn oil (Fig. 4C). Dietary medium-chain triglycerides prevented these pathological changes nearly completely (Fig. 4D). Hepatic pathology is summarized in Fig. 5. Histology was normal in rats fed corn oil or medium-chain triglycerides without ethanol. In contrast, rats fed corn oil with ethanol developed moderate fatty infiltration and slight inflammation after 2 wk. On the other hand, rats fed medium-chain triglycerides with ethanol had essentially no liver injury, confirming earlier work (36).

Effect of chronic ethanol and lipid type on LPS-induced increases in \([\text{Ca}^{2+}]_i\) in isolated Kupffer cells. \([\text{Ca}^{2+}]_i\) in isolated Kupffer cells from rats fed ethanol with corn oil or medium-chain triglycerides was assessed fluorometrically using fura 2 as described in MATERIALS AND METHODS. Representative traces are shown.

<Fig. 6. Effect of chronic ethanol and lipid type on lipopolysaccharide (LPS)-induced increase in intracellular \([\text{Ca}^{2+}]_i\) in isolated Kupffer cells. \([\text{Ca}^{2+}]_i\) in isolated Kupffer cells from rats fed ethanol with corn oil or medium-chain triglycerides was assessed fluorometrically using fura 2 as described in MATERIALS AND METHODS. Representative traces are shown.>

**Fig. 5.** Effect of chronic ethanol and lipid type on hepatic pathology score. Pathology was scored as described in MATERIALS AND METHODS. Steatosis and inflammation are shown individually. Data represent means \pm SE \((n = 6\) rats). *\(P < 0.05\) compared with rats fed corn oil without ethanol and \#P < 0.05 compared with rats fed corn oil with ethanol by Mann-Whitney rank-sum test.
nels (21), and [Ca\textsuperscript{2+}] plays an important role in activation of Kupffer cells (13). Accordingly, [Ca\textsuperscript{2+}] in isolated Kupffer cells was measured. After addition of LPS (10 µg/ml) to Kupffer cells from rats fed corn oil with ethanol for 2 wk, [Ca\textsuperscript{2+}] increased rapidly from basal values around 10 nM to a maximal values of 338 nM within 60 s (Fig. 6). This increase was blunted ~50% in cells from rats fed medium-chain triglycerides with ethanol.

In cultured Kupffer cells from rats fed corn oil with ethanol, the increase in [Ca\textsuperscript{2+}], caused by LPS was dose dependent, with maximal responses observed with 10 µg/ml LPS (Fig. 7). In cells from rats fed medium-chain triglycerides with ethanol, however, the increase in [Ca\textsuperscript{2+}] caused by LPS was blunted by ~50% at all concentrations studied.

Effect of chronic ethanol and lipid type on TNF-α production by isolated Kupffer cells. In isolated Kupffer cells from rats fed corn oil with ethanol, TNF-α production caused by LPS was dose dependent, with maximal responses observed with 10 µg/ml LPS (Fig. 8). In cells from rats fed medium-chain triglycerides with ethanol, however, values were blunted significantly by ~40% at the two higher concentrations studied.

Effects of chronic ethanol and lipid type on free radical formation. Radical adducts were barely detectable in bile from rats fed an ethanol-free, high-fat control diet in both groups (data not shown). After enteral ethanol for 2 wk, however, POBN radical adducts were about twofold greater in corn oil-treated than in medium-chain triglyceride-treated rats (Fig. 9). Computer simulations of these spectra are shown in Fig. 9. Coupling constants were \( a^N = 15.70 \) G and \( a^H = 2.72 \) G. Average ESR signal intensity was measured as the double integral of the two low-field peaks of each spectrum (Fig. 10). Although ethanol treatment significantly increased the intensity of these signals in both groups, the average intensity from rats fed corn oil was about twofold greater than values from rats fed medium-chain triglycerides.

Effects of chronic ethanol on CD14 expression on the Kupffer cell. After 2 wk of a high-fat control diet with corn oil or medium-chain triglycerides, expression of the endotoxin receptor CD14 on Kupffer cells was low; however, enteral ethanol with corn oil increased CD14 expression significantly about threefold (Fig. 11). These increases were blunted significantly to a value similar to that of controls by dietary medium-chain triglycerides.

**DISCUSSION**

Effect of dietary medium-chain triglycerides on plasma endotoxin levels. Gram-negative bacterial species are a major source of endotoxin in the gut microflora (9). Alcohol modifies gut flora (10) and increases gut permeability to normally nonabsorbed substances, leading to an increase of blood endotoxin levels (8). Indeed, gut permeability was increased after 2 wk of ethanol in this study (Fig. 3). Blood endotoxin levels began to increase after 2–4 wk of ethanol treatment in the Tsukamoto-French model, and a good correlation between blood
endotoxin and pathology has been observed (2). Previous work has shown that intestinal sterilization with antibiotics (2) and suppression of endotoxin production with lactobacillus feeding (34) minimized early alcohol-induced liver injury in the Tsukamoto-French model. Furthermore, destruction of Kupffer cells by GdCl3 prevented liver injury (1). These data are consistent with the hypothesis that Kupffer cells activated by gut-derived endotoxin are involved in the mechanism of early alcohol-induced liver injury (43).

Nutrition and dietary factors are important in the pathogenesis of alcohol-induced liver disease (16). It is known that saturated fat prevents early alcohol-induced liver injury; however, mechanisms have remained unclear (35, 38). It has been reported that medium-chain triglycerides change brush-border structure in the small intestine (48) and may affect gut permeability or gut microflora. Indeed, the increase of gut permeability caused by enteral ethanol was blunted significantly by medium-chain triglycerides (Fig. 3). Furthermore, dietary medium-chain triglycerides prevented injury of the intestine caused by enteral ethanol with corn oil (Fig. 4). Importantly, medium-chain triglycer-
Fig. 12. Scheme depicting working hypothesis for effect of medium-chain triglycerides on early alcohol-induced liver injury. Endotoxin activates Kupffer cells to release inflammatory mediators such as TNF-α, eicosanoids, and free radicals. Increase of endotoxin levels after ethanol exposure is blunted by medium-chain triglycerides. It is possible that medium-chain triglycerides affect microflora in the gut, gut permeability to endotoxin, or endotoxin clearance. Moreover, because medium-chain triglycerides blunt increase in [Ca^{2+}], and production of TNF-α caused by LPS in isolated Kupffer cells, it is likely that medium-chain triglycerides also alter signaling cascade triggered by LPS binding to receptors on Kupffer cells, possibly by altering membrane structure.

Endotoxin activates Kupffer cells to release inflammatory mediators such as TNF-α, eicosanoids, and free radicals. Increase of endotoxin levels after ethanol exposure is blunted by medium-chain triglycerides. It is possible that medium-chain triglycerides affect microflora in the gut, gut permeability to endotoxin, or endotoxin clearance. Moreover, because medium-chain triglycerides blunt increase in [Ca^{2+}], and production of TNF-α caused by LPS in isolated Kupffer cells, it is likely that medium-chain triglycerides also alter signaling cascade triggered by LPS binding to receptors on Kupffer cells, possibly by altering membrane structure.

Role of endotoxin and Kupffer cells in early alcohol-induced liver injury. The major target of endotoxin is the Kupffer cell (30). Endotoxin activates Kupffer cells via the endotoxin receptor CD14, which is on the plasma membrane in Kupffer cells. Activated Kupffer cells release mediators, including cytokines, eicosanoids, and free radicals, which induce liver injury. Because destruction of Kupffer cells by GdCl3 prevented liver injury in the Tsukamoto-French model (1), it was proposed that activation of Kupffer cells by endotoxin was responsible for alcohol-induced liver injury. CD14 is upregulated in Kupffer cells from rats given acute and enteral ethanol (15, 41). Furthermore, medium-chain triglyceride feeding for 1 wk blunted CD14 expression on the Kupffer cell (Fig. 11), confirming previous work by Su et al. (41). Moreover, medium-chain triglycerides significantly blunted increases in intracellular Ca^{2+} caused by LPS in isolated Kupffer cells (Figs. 6 and 7). Thus medium-chain triglycerides blunt the response of Kupffer cells to endotoxin, in part, by inhibition of expression of the endotoxin receptor CD14.

Ca^{2+} is essential for activation of Kupffer cells (26), which contain voltage-dependent Ca^{2+} channels (21). Nimodipine, a dihydropyridine-type Ca^{2+} channel antagonist, prevented alcoholic liver injury in the Tsukamoto-French model (25). These data suggest that [Ca^{2+}], plays an important role in mechanisms of alcohol-induced liver injury. A transient increase of [Ca^{2+}], is required for LPS-induced expression of TNF-α (49), which plays a pivotal role in the inflammatory cytokine cascade (30). TNF-α stimulates endothelial cells to synthesize adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1), that induce infiltration of neutrophils in the liver and cause microcirculatory disturbances leading to liver injury (51). Indeed, ethanol increased TNF-α mRNA and ICAM-1 expression in the liver in the Tsukamoto-French model (23). Furthermore, anti-TNF-α antibody reduced inflammatory cell infiltration and necrosis in the liver in this model (24). Moreover, early alcohol-induced liver injury was diminished in TNF-α receptor-1 knockout mice treated with enteral ethanol (50). Thus evidence continues to accumulate indicating that TNF-α plays an important role in alcohol-induced liver injury. Indeed, Nanji et al. (38) reported that medium-chain triglycerides decreased TNF-α mRNA expression in the liver in the Tsukamoto-French model. Here, the increase of TNF-α production in isolated Kupffer cells from rats fed ethanol was decreased significantly by medium-chain triglycerides (Fig. 8). Thus medium-chain triglycerides are most likely protective by reducing TNF-α production.

Role of hypoxia and free radicals. Alcohol causes a hypermetabolic state that could cause hypoxia in the liver (47). Moreover, Kupffer cells from rats given ethanol produce eicosanoids that stimulate parenchymal cell oxygen metabolism, leading to hypoxia and free radical formation (40). Indeed, hypoxia at the tissue level detected with 2-nitroimidazole hypoxia markers and free radicals in bile detected by spin trapping and ESR are hallmarks of the Tsukamoto-French model (3). Moreover, destruction of Kupffer cells with GdCl3 diminished free radical formation and prevented liver injury in this model. In the present study, free radical formation was blunted significantly by dietary medium-chain triglycerides (Figs. 9 and 10). These data are consistent with the hypothesis that hypoxia after ethanol and free radical formation by Kupffer cells are involved in early alcohol-induced liver injury.

In conclusion, it is proposed that medium-chain triglycerides prevent early alcoholic liver injury by inhibition of free radical formation in the liver and TNF-α production caused by endotoxin-mediated activation of Kupffer cells. This likely involves effects on both the gut and the cell membrane structure of Kupffer cells, leading to both decreased plasma endotoxin levels and blunted responsiveness of Kupffer cells to gut-derived endotoxin (see Fig. 12).

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