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Preservation of hepatocyte nuclear factor 4α contributes to the beneficial effect of dietary medium chain triglyceride on alcohol-induced hepatic lipid dyshomeostasis in rats

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Abstract

Background—Alcohol consumption is a major cause of fatty liver, and dietary saturated fats have been shown to protect against alcoholic fatty liver. This study investigated the mechanisms of how dietary saturated fat may modulate alcohol-induced hepatic lipid dyshomeostasis.

Methods—Rats were pair-fed with 3 isocaloric liquid diets, control, alcohol, and medium chain triglyceride (MCT)/alcohol, respectively, for 8 weeks. The control and alcohol diets were based on the Lieber-DeCarli liquid diet formula with 30% total calories derived from corn oil (rich in unsaturated long chain fatty acids). The corn oil was replaced by MCT, which consists of exclusive saturated fatty acids, in the MCT/alcohol diet. HepG2 cell culture was conducted to test the effects of unsaturated fatty acids on HNF4α and the role of HNF4α in regulating hepatocyte lipid homeostasis.

Results—Alcohol feeding caused significant lipid accumulation, which was attenuated by dietary MCT. The major effect of alcohol on hepatic gene expression is the up-regulation of CYP4A1, CD36 and GPAT3, and down-regulation of apolipoprotein B (ApoB). Dietary MCT further up-regulated CYP4A1 gene, normalized ApoB gene and up-regulated MTTP and SCD1 genes. The protein level of hepatocyte nuclear factor-4α (HNF4α), a master transcription factor of the liver, was reduced by alcohol feeding, which was normalized by dietary MCT. Fatty acid profiling demonstrated that alcohol feeding dramatically increased hepatic unsaturated long chain fatty acyl species, particularly linoleic acid and oleic acid, which was attenuated by dietary MCT. Dietary MCT attenuated alcohol-reduced serum triglyceride level and modulated the fatty acid composition of the serum triglycerides. Cell culture study demonstrated polyunsaturated linoleic acid rather than monounsaturated oleic acid inactivated HNF4α in HepG2 cells. Knockdown HNF4α caused lipid accumulation in HepG2 cells due to dysregulation of very low density lipoprotein secretion.

Conclusions—Results suggest that dietary MCT prevents alcohol-induced hepatic lipid accumulation, at least partially, through reducing hepatic polyunsaturated long chain fatty acids and preserving HNF4α.

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Keywords

Alcoholic fatty liver; Corn oil; Medium chain triglyceride; Lipid metabolism; HNF4α

Introduction

Excess alcohol consumption is a major etiologic factor in the development of fatty liver (steatosis). Alcoholic steatosis is characterized by the accumulation of large lipid droplets (macrovesicle steatosis) in hepatocytes (Lakshman, 2004; Purohit et al., 2009). Mechanistic studies have shown that alcohol consumption could affect multiple hepatic lipid metabolic pathways, including lipid uptake, *de novo* lipogenesis (DNL), fatty acid oxidation and very low-density lipoprotein (VLDL) export, thereby resulting in lipid deposition in the liver (Sozio and Crabb, 2008; Sozio et al., 2010). Although liver steatosis was initially considered benign, increasing evidence suggest that excess fat accumulation plays a direct role in the progression to more advanced alcoholic liver injury, with the severity of damage proportional to the extent of steatosis (Gyamfi and Wan, 2010; Nagy, 2004; Sorensen et al., 1984). Alcoholic steatosis is reversible and prevention or reduction of steatosis during alcohol consumption may block or delay the progression of alcoholic liver disease.

Fatty acid composition of dietary fat can influence the relative proportions of fatty acids in various tissues (Jones et al., 1995). Moreover, fatty acids with different chain length, degree of unsaturation, and position of double bonds may regulate signaling pathways, thereby producing diverse metabolic outcomes (Moussavi et al., 2008; Tovar et al., 2011). Several lines of evidence have shown that dietary fat content and composition play an important role in the pathogenesis of alcoholic liver disease (Mezey, 1998; Nanji, 2004). Pair-feeding (equal daily calorie intake) studies have demonstrated that polyunsaturated fat potentiates, while saturated fat such as cocoa butter and medium chain triglycerides (MCT) prevents alcohol-induced liver injury in rodents (Liang et al., 2011; Nanji et al., 2001; Polavarapu et al., 1998; Ronis et al., 2004; You et al., 2005, You et al., 2008,); this is obviously controversial to the observations in non-alcoholic liver disease (Zelber-Sagi et al., 2011). Previous studies have shown that activations of SIRT1-AMPK pathway and fatty acid oxidation were associated with the benefits of saturated fat (Liang et al., 2011; Nanji, 2004; Ronis et al., 2004). However, the mechanisms of how dietary fat sources differentially modulate hepatic lipid homeostasis have not been fully defined.

Hepatocyte nuclear factor-4α (HNF4α, NR2A1) is a highly conserved member of the nuclear receptor superfamily, and plays an important role in early development (Hayhurst et al., 2001). HNF4α is a master regulator of liver gene expression, and critically regulates multiple metabolic pathways (Odom et al., 2004; Watt et al., 2003). Liver-specific conditional knockout of HNF4α in adult mice led to severe steatosis in association with disruption of very low density lipoprotein (VLDL) secretion (Hayhurst et al., 2001). In our previous studies, we have found that chronic ethanol exposure inactivates HNF4α, which contributes to the pathogenesis of alcohol-induced cell death and lipid dyshomeostasis in the liver (Kang et al., 2008; Kang et al., 2009). Moreover, HNF4α activity may be modulated by fatty acyl-coenzyme A (CoA) thioesters, which may act as agonistic or antagonistic ligands depending on chain length and degree of saturation (Hertz et al., 1998). Here we report that dietary saturated fat abrogates alcohol-induced alterations of hepatic fatty acid profile and HNF4α which critically regulates lipid homeostasis in hepatocytes.

Materials and methods

Animals and alcohol feeding experiments

Male Sprague Dawley rats were obtained from Charles River (Wilmington, MA) and treated according to the experimental procedures approved by the Institutional Animal Care and Use Committee. Rats at 3 months old were pair-fed with 3 isocaloric liquid diets, control, alcohol, and MCT/alcohol, respectively, for 8 weeks. The control and alcohol diets were prepared based on the most widely used Lieber-DeCarli liquid diet formula (Lieber et al., 1989) with 30% total calories derived from corn oil (rich in long chain unsaturated fatty acids). The corn oil was replaced by MCT (Exclusively saturated fatty acids) in the MCT/alcohol diet. Safflower oil was used as source of essential fatty acids. All gradients for the liquid diets were obtained from Dyets (Bethlehem, PA), except that 95% ethanol was obtained from Sigma-Aldrich (St. Louis, MO). The compositions of dietary calories of the liquid diets are listed in Table 1, and the fatty acid compositions of corn oil and MCT are shown in Table S1. Because MCT/alcohol-fed rats had lowest food intake, the other two groups were pair-fed the amount that MCT/alcohol-fed rats had in the previous day. The ethanol content (%, w/v) was gradually increased from 5% for the first 2 weeks to 5.6% for the last 2 weeks, increasing by 0.2% every 2 weeks. At the end of the experiment, rats were anesthetized with isofluorane after 4-hr fasting, and serum and liver tissue samples were harvested for assays.

HepG2 Cell Culture

HepG2 cells obtained from American Type Culture Collection (Rockville, MD) were grown in Dulbecco's modified Eagle medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and penicillin (100 U/ml)/streptomycin (100 U/ml) (Invitrogen). To examine the effects of unsaturated fatty acids on HNF4α, cells were treated with either polyunsaturated linoleic acid or monounsaturated oleic acid. Linoleic acid-BSA (LA-BSA) and oleic acid-BSA (OA-BSA) complexes were prepared by conjugating linoleic acid sodium salt or sodium oleate (Sigma-Aldrich) with 5% fatty acid-free BSA in phosphate-buffered saline at 6.6:1 molar ratio. HepG2 cells were treated with LA-BSA or OA-BSA at 0, 0.25, 0.5 or 1.0 mM for 6 hr or 24 hr. For HNF4α knockdown, HepG2 cells were transfected with Silencer predesigned human HNF4α siRNA and Negative Control #1 siRNA (Ambion, Austin, TX) using Lipofectamine 2000 transfection reagent (Invitrogen) following the manufacturer's instructions. Three days after siRNA transfection, cells and media were collected for analysis.

Blood Metabolites Assay

Blood glucose was measured using OneTouch Ultra2 blood glucose meter (Life Scan, Milpitas, CA). Serum triglyceride and cholesterol were measured using the Infinity Assay Reagents (Thermo Scientific, Waltham, MA). Serum free fatty acids (FFA) were determined with a BioVision FFA Kit (Mountain View, CA). Serum β-hydroxybutyrate (ketone body) was determined using the Cayman Chemical β-hydroxybutyrate assay kit (Ann Arbor, MI). Serum ethanol concentration was measured using Biovision ethanol assay kit.

Determination of lipid accumulation

Liver tissues were frozen in Tissue-Tek OTC (Optimum cutting temperature) Compound (VWR, Batavia, IL) and cryostat tissue sections were cut at 7 μm. Both cryostat liver tissue sections and HepG2 cells on chamber slides were fixed with 10% formalin for 10 min, and processed for the staining of neutral lipid with Oil Red O solution. Quantitative assay of lipids was conducted by measuring the concentrations of triglycerides, cholesterol esters and free fatty acids in liver tissues and hepatocytes using BioVision Assay kits.

Lipid profiling

The hepatic and serum lipid profiles were analyzed by a combination of thin-layer chromatography (TLC) and gas chromatography/mass spectrometry system (GC/MS) as previously described (Puri et al., 2007). Briefly, Total lipids were extracted from 20mg of liver tissue or 380μl of serum by Folch's method with chloroform/methanol (2:1vol/vol). Glyceryl triheptadecanoate (C17:0, TG, 100μg), Tricosanoic acid (C23:0, FFA, 100μg), and 1,2-dinonadecanoyl-*sn*-glycero-3-phosphocholine (C19:0, PC, 100μg) were added as internal standards. Sample extracts were dried under nitrogen, reconstituted with chloroform and spotted onto silica Gel 60 TLC plates (Merck, Darmstadt, Germany). TLC plates were first developed with chloroform/methanol/acetic acid/water (100:67:7:4 by volume) solvent system to separate the phospholipids, and then developed with hexane/diethyl ether/acetic acid (80:20:1.5 by volume) mobile phase to separate each of the simple lipids. Two standard lanes consisting of authentic triglyceride, FFA and phospholipids were spotted on the outside lanes of the TLC plate as reference compounds.

After visualized by spraying with 20% ethanolic phosphomolybdic acid, the triglyceride, FFA and total phospholipids spots were scraped and transesterified in 3N methanolic HCl under an N_2 atmosphere at 100°C for 45 minutes in a sealed vial. The resulting fatty acid methyl esters were extracted with hexane containing 0.05% butylated hydroxytoluene and prepared for gas chromatography through the sealing of the hexane extracts under nitrogen. Fatty acid methyl esters were separated and quantified by a Pegasus HT time-of-flight mass spectrometer (Leco Corporation, MI) equipped with a 30-m stabilwax capillary column (Restek, PA, USA), with helium as the carrier gas at a constant flow rate of 1.0 mL/min. The temperature of injection, transfer interface, and ion source was set to 250 °C, 210 °C, and 210 °C, respectively. GC oven temperature was start 120 °C for 2 min, followed by 10 °C/min to 180 °C, 4 °C/min to 230 °C, and kept for 10 min at 230 °C. The mass range was set at 35-600, with an acquisition rate of 10 spectrum/s. The acquired data was analyzed by ChromTOF software (v 4.22, Leco).

qRT-PCR analysis

Total RNA was isolated from liver tissues, and reverse transcribed with TaqMan Reverse Transcription Reagents (Life Technologies, Carlsbad, CA). The forward and reverse primers were purchased from Integrated DNA Technologies (Coralville, IA). *q*RT-PCR analysis with SYBR green PCR Master Mix (Qiangen, Valencia, CA) was performed on an Applied Biosystems PRISM 7500 Sequence Detection System (Applied Biosystems). Primer sets used are showed in Table S2. The data were normalized to 18S rRNA mRNA expression and presented as fold changes, setting the values of control as one.

Immunohistochemical Staining of HNF4α

Liver tissue sections were rehydrated and incubated overnight at 4°C with a polyclonal rabbit anti-HNF4α (Santa Cruz Biotechnologies, Santa Cruz, CA), followed by incubation with DAKO EnVision$^+$ peroxidase labeled polymer conjugated to goat anti-rabbit antibody (DAKO, Carpinteria, CA) for 30 minutes. Visualization was conducted using diaminobenzidine as HRP substrate.

Immunoblot analysis

Liver tissue or HepG2 cell proteins (50μg protein) were loaded onto 4-12% sodium dodecyl sulfate-polyacrylamide gel. After electrophoresis, proteins were transferred to PVDF membranes. The membrane was probed with rabbit polyclonal or mouse monoclonal antibodies against CYP2E1 from Calbiochem (San Diego, CA), ADH (alcohol dehydrogenase) and HNF4α from Novus Biotechnologies (Littleton, CO), and ApoB100

(apolipoprotein B 100), ACADM (acyl-CoA dehydrogenase, medium chain), ACADL (acyl-CoA dehydrogenase, long chain), MTTP (microsomal triglyceride transfer protein), HNF1α (hepatocyte nuclear factor-1α), PPARα (peroxisome proliferator-activated receptor α), and C/EBPα (CCAAT/enhancer-binding protein α) from Santa Cruz Biotechnologies (Santa Cruz, CA), followed by incubation with HRP-conjugated goat anti-rabbit IgG, or goat anti-mouse IgG antibody Protein bands were visualized by an Enhanced Chemiluminescence detection system (GE Healthcare, Piscataway, NJ) and quantified by densitometry analysis.

Statistical analysis

Results of lipid profiles are expressed as mean ± SEM, while all the other results are expressed as mean ± SD. Data of the animal study and cell culture study of unsaturated fatty acids on HNF4α expression were analyzed by one way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. Data of the HNF4α knockdown cell culture study were analyzed by independent samples *t*-test. Differences between groups were considered significant at $p < 0.05$.

Results

Body weight, liver weight, blood parameters and alcohol metabolism of rats after 8 weeks of liquid diet feeding

As shown in Table 2, there were no significant differences in final body weight among the treatments. However, the liver weight was increased in rats fed alcohol or MCT/alcohol in comparison with the controls. Alcohol feeding decreased blood glucose level and increased serum β-hydroxybutyrate level, and dietary MCT did not correct these alterations. Alcohol feeding increased hepatic CYP2E1 protein level, but did not affect ADH protein level (Figure 1A). MCT/alcohol group showed same effects on hepatic CYP2E1 and ADH as alcohol group. Accordingly, the blood alcohol levels were similar in alcohol and MCT/alcohol groups (Figure 1B).

Dietary MCT prevented alcohol-induced lipid accumulation in the liver of rats

Oil red O staining demonstrated that alcohol feeding caused significant accumulation of large lipid droplets in the liver, while only tiny lipid droplets were found in the liver of control and MCT/Alcohol groups (Fig. 2A). Quantitative measurements of hepatic lipid contents corresponded with the histological analysis. Alcohol feeding remarkably increased hepatic concentrations of triglyceride (~5 fold), cholesterol esters (~3 fold), and FFA; all these alterations were normalized by dietary MCT (Fig. 2B). Hepatic concentration of phospholipids was not affected by alcohol feeding with or without dietary MCT.

Dietary MCT attenuated alcohol-induced alterations in fatty acid profile of hepatic lipids in rats

The fatty acid profiles of hepatic total lipids, triglycerides, phospholipids and FFA were analyzed by GC/MS after TLC separation. As shown in Table 3, 21 fatty acyl species were detected in total lipids, and alcohol feeding increased 16 fatty acyl species, among which, 12 fatty acyl species were unsaturated and 4 saturated. Dietary MCT attenuated alcohol-increased fatty acyl species of total lipids except myristic acid (C14:0) and stearic acid (C18:0), whereas it increased the medium chain fatty acids, caprylic acid (C8:0), capric acid (C10:0) and lauric acid (C12:0). Fatty acid profile of hepatic triglycerides was similar to that of total lipids (Table 4). Alcohol feeding increased 16 fatty acyl species, and dietary MCT normalized alcohol-increased all unsaturated fatty acyl species except palmitoleic acid (C16:1 n7). Dietary MCT also attenuated alcohol-increased saturated fatty acyl species except myristic acid (C14:0). In addition, dietary MCT increased the medium chain fatty

acyl species in hepatic triglycerides compared to the control and alcohol groups, including caprylic acid (C8:0), capric acid (C10:0) and lauric acid (C12:0).

Table 5 shows hepatic FFA profile. The major effect of alcohol feeding on hepatic FFA is the significant increase in oleic acid (C18:1 n9) and linoleic acid (C18:2 n6). Dietary MCT reduced hepatic oleic acid (C18:1 n9) and linoleic acid (C18:2 n6) to a level even lower than the control group. On the other hand, dietary MCT significantly increased the medium chain free fatty acids, caprylic acid (C8:0) and capric acid (C10:0).

Dietary MCT upregulated hepatic expression of genes involved in fatty acid oxidation and VLDL secretion in rats

To explore the mechanisms of how MCT ameliorated alcohol-induced hepatic lipid dyshomeostasis, hepatic genes related to fatty acid oxidation, VLDL secretion, fatty acid transport, fatty acid synthesis and triglyceride synthesis measured and data are shown in Figure 3. Among the 5 genes involved in fatty acid oxidation, alcohol feeding only up-regulated CYP4A1, and dietary MCT further promoted CYP4A1 expression (Fig. 3A). MCT down-regulated CPT1A and ACADL, which are not required for MCT oxidation. Among the VLDL assembly genes, alcohol feeding significantly down-regulated ApoB mRNA expression (Fig. 3B). Dietary MCT significantly increased the mRNA level of MTTP and normalized that of ApoB. Among genes involved in fatty acid uptake/transport, only CD36 (fatty acid translocase) was up-regulated by alcohol feeding regardless of dietary MCT (Fig. 3C). Fatty acid synthesis genes were not affected by alcohol feeding, but dietary MCT up-regulated FAS (Fatty acid synthase) and SCD1 (Stearoyl-CoA desaturase 1), which are important DNL enzymes (Fig. 3D). Among 7 genes involved in triglyceride synthesis, GPAT3 (glycerol-3-phosphate acyltransferase 3) was most significantly affected, a 4-fold increase in mRNA level, by both alcohol and MCT/alcohol feeding. Dietary MCT also up-regulated GPAT1. However, GPAT2 was down-regulated by both alcohol and MCT/alcohol feeding.

Dietary MCT prevented alcohol-reduced HNF4α protein in the liver of rats

HNF4α is a master transcription factor in regulation of lipid metabolism, and its activity could be regulated by unsaturated (antagonist ligands) and saturated (agonist ligands) fatty acids. Thus, alterations of HNF4α in the liver were assessed by immunohistochemical staining and immunoblot analysis. Immunohistochemical staining showed HNF4α mainly locates in the nuclei, and a reduced nuclear distribution, particularly in hepatocytes with lipid droplet accumulation in the liver of alcohol-fed rats; which was attenuated by dietary MCT (Fig. 4A). Immunoblot analysis also demonstrated that alcohol feeding reduced, while dietary MCT normalized, the protein level of HNF4α in the liver (Fig. 4B).

Dietary MCT attenuated alcohol-induced alterations of serum lipid concentrations and fatty acid profile of serum lipids in rats

As shown in Table 6, alcohol feeding decreased serum triglyceride concentration, increased serum cholesterol concentration, but did not affect serum FFA level. Dietary MCT normalized serum triglyceride and cholesterol levels and elevated serum FFA level. The fatty acyl species of the total serum lipids were not affected by alcohol feeding, but 4 fatty acyl species were increased and 4 were reduced by dietary MCT (Table S3). Analysis of the fatty acyl composition of serum triglycerides demonstrated that alcohol feeding significantly reduced myristic acid (C14:0) and palmitic acid (C16:0). Dietary MCT significantly increased several fatty acyl species of triglycerides, including caprylic acid (C8:0), capric acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1 n7) and stearic acid (C18:0). Among these increased fatty acids, the last 4 components are apparently biosynthesized in the liver. In contrast, dietary MCT decreased

linoleic acid (C18:2 n6) (Table 6). The serum FFA profile was not affected by alcohol feeding, but dietary MCT increased caprylic acid (C8:0) and capric acid (C10:0) (Table S4).

Effects of unsaturated fatty acids on HNF4α in HepG2 cells

To test the effects of unsaturated fatty acids on hepatocyte HNF4α, HepG2 cells were treated with polyunsaturated linoleic acid (C18:2) or monounsaturated oleic acid (C18:1), which are the major unsaturated fatty acids accumulated in the liver in alcohol-fed mice. As shown in Figure 5, linoleic acid at either 0.25, 0.5 or 1.0 mM did not affect the HNF4α protein level at 6 hr, but significantly reduced the HNF4α protein levels at 24 hr. In contrast, oleic acid at 0.5 or 1.0 mM increased the HNF4α protein level at 6 hr, but this effect disappeared at 24 hr.

Knockdown of HNF4α led to cellular lipid accumulation in association with an impaired ApoB100 secretion in HepG2 cells

To define the role of HNF4α in lipid homeostasis, HepG2 cells were transfected with HNF4α siRNA. Immunoblot analysis demonstrated that HNF4α siRNA transfection decreased HNF4α protein level without significantly affecting other major transcription factors involved in lipid metabolism, including HNF1α, PPARα and C/EBPα (Fig. 6A). Importantly, HNF4α siRNA transfection remarkably suppressed the cellular protein levels of MTTP, ApoB100, and ACADL (Fig. 6B). HNF4α siRNA transfection caused lipid accumulation in the HepG2 cells as indicated by the presence of lipid droplets in the cytoplasm (Fig. 7A) and the increased concentrations of cellular triglycerides and FFA (Fig. 7B). In accordance, HNF4α siRNA transfection reduced ApoB100 secretion to the media (Fig. 7C).

Discussion

Alcohol is metabolized primarily in the liver, and oxidation of alcohol by alcohol dehydrogenase and acetaldehyde by aldehyde dehydrogenase cause an increase in NADH/NAD^+ ratio. This shift of the redox state in mitochondria inhibits other NADH-producing reactions, especially the α-ketoglutarate dehydrogenase reaction in the citric acid cycle. By this means, fatty acid oxidation via β-oxidation and the citric acid cycle is inhibited, and ketogenesis is induced (French, 1989; McGuire et al., 2006; Sozio and Crabb, 2008). Furthermore, ethanol treatment stimulates adipose lipolysis and promotes fatty acid flux to the liver (French, 1989; McGuire et al., 2006). In the present study, we found that serum β-hydroxybutyrate concentration, hepatic GPAT3 and CD36 mRNA levels were remarkably increased in both alcohol and MCT/alcohol groups. Increased serum β-hydroxybutyrate concentration indicates a blockage of the entrance of acyl-CoA to citric acid cycle for a full oxidation to NADH and FADH2 which are fed into the oxidative phosphorylation pathway for ATP production (McGuire et al., 2006). GPAT3, an enzyme in endoplasmic reticulum, catalyzes the initial step in triglyceride synthesis through the acylation of glycerol 3-phosphate (Takeuchi and Reue, 2009). CD36 is a transporter mediating the liver fatty acid uptake from the circulation (Clugston et al., 2011). These data demonstrated that decreased mitochondrial fatty acid oxidation via the citric acid cycle, increased tendency of triglyceride synthesis and increased transport of fatty acids from adipose tissue to the liver are induced in both alcohol and MCT/alcohol rats.

Different fatty acids have different metabolic fate after absorption, for example, oleic acid and linoleic acid are incorporated mainly into triglyceride, while stearic acid and arachidonic acid are used preferentially for phospholipid synthesis (Kritchevsky, 1994; Listenberger et al., 2003; Wang and Koo, 1993). Medium chain fatty acids are rarely incorporated into liver lipids but oxidized or elongated in the liver (Papamandjaris et al., 1998; Wang and Koo,

1993). The possible reason for this diversity is that enzymes for lipid metabolism may have different preference for fatty acid species (Takeuchi and Reue, 2009). However, lipid fatty acid spectra analysis of hepatic total lipids and triglycerides in the present study showed that MCT increased fatty acids with carbon number less than C16, but did not affect that with carbon number more than C18 except C20. We also found that in MCT feeding rats, through DNL and chain elongation, several long chain fatty acids were produced which are needed to be incorporated into liver phospholipids and triglycerides. These results demonstrated that different dietary fats may affect hepatic lipid metabolism and composition.

Alcohol exposure may impair mitochondrial fatty acid β-oxidation (French, 1989; Sozio and Crabb, 2008). However, inhibition of mitochondrial oxidation could result in enhanced oxidation of fatty acids by extra-mitochondrial pathways, particularly the ω-oxidation in the endoplasmic reticulum. Fatty acid ω-oxidation is catalyzed mainly by cytochromes P450 4A subfamily and produce water soluble dicarboxylic acid (Christensen et al., 1991; Nanji et al., 2004). Previous studies have indicated that ethanol consumption enhances fatty acid ω-oxidation along with the increase in hepatic CYP4A1 expression (Ma et al., 1993; Nanji et al., 2004; Ronis et al., 2004). Moreover, fatty acid ω-oxidation may be structurally specific, and medium chain fatty acids such as lauric acid and capric acid are likely the major substrates for this oxidative pathway (Christensen et al., 1991; Papamandjaris et al., 1998). The present study showed that hepatic genes related to mitochondrial fatty acid ω-oxidation were not affected by alcohol feeding, but MCT/alcohol feeding down-regulated hepatic CPT1A and ACADL genes which are not required for mitochondrial ω-oxidation of MCT (Papamandjaris et al., 1998). On the other hand, hepatic CYP4A1 gene was significantly up-regulated by both alcohol and MCT/alcohol feeding, with a greater value in the later. However, MCT did not affect alcohol-elevated serum β-hydroxybutyrate, a metabolite from fatty acid β-oxidation. These results indicate that MCT impacts fatty acid ω-oxidation but not β-oxidation. Because mitochondria are the major organelle metabolizing fatty acids, the data on fatty acid oxidation cannot well explain the MCT effects on hepatic lipid accumulation.

Chronic alcohol exposure has been shown to markedly inhibit VLDL synthesis and secretion, which has been implicated in the pathogenesis of alcoholic fatty liver (Lakshman et al., 1989; Venkatesan et al., 1988). In the present study, alcohol feeding significantly decreased serum triglyceride level, indicating a reduced VLDL export from the liver. Surprisingly, MCT/alcohol-fed rats showed a normal serum triglyceride level. This tendency can also be reflected by the fatty acid profile of serum triglycerides. It is well known that triglycerides exist mainly in two forms of lipoproteins in the circulation: liver-derived VLDL and intestine-derived chylomicrons (Xiao et al., 2011). Unlike long chain triglycerides, MCT are hydrolyzed faster and more completely. After absorption by the intestinal epithelium, long chain fatty acids are preferentially incorporated into chylomicrons and transported into the circulation via lymph, whereas medium chain fatty acids, upon binding to albumin are directly transported to the liver via the portal vein (Bach and Babayan, 1982; Papamandjaris et al., 1998). These data, combined with our observation that fatty acyl composition of serum triglycerides in MCT/alcohol-fed rats resembled those of liver, indicated that serum triglycerides of MCT/alcohol-fed rats mainly comes from VLDL. Therefore, MCT/alcohol-fed rats had significantly higher VLDL secretion than alcohol-fed rats. Indeed, we also found that alcohol feeding significantly down-regulated ApoB mRNA expression. Dietary MCT significantly increased mRNA levels of ApoB as well as MTTP, key molecules in VLDL synthesis and secretion (Xiao et al., 2011). Taken together, the present study indicated that dietary MCT may prevent alcoholic steatosis by increasing hepatic VLDL secretion.

HNF4α critically regulates hepatic lipid homeostasis (Watt et al., 2003). Conditional liver-specific disruption of HNF4α in mice led to severe steatosis and reduced serum triglyceride level, which was associated with a selective disruption of ApoB and MTTP gene expression (Hayhurst et al., 2001). HNF4α activity can be regulated by fatty acyl-CoAs (Hertz et al., 1998). Unsaturated long-chain fatty acyl-CoAs such as (C18:2 n6)-CoA, (C18:3 n3)-CoA and (C20:5, n3)-CoA suppress HNF4α transcriptional activity, while saturated fatty acyl-CoAs with chain lengths of 14 and 16 carbon atoms positively regulate HNF4α activity (Hertz et al., 1998; Petrescu et al., 2002). Fatty acyl profiles of liver free fatty acid and liver total lipids in this study demonstrated accumulation of unsaturated long-chain fatty acids, particularly linoleic acid (C18:2) and oleic acid (C18:1) in the liver of alcohol-fed rats, whereas MCT/alcohol-fed rats showed more medium chain and newly produced saturated (C16:0) and monounsaturated fatty acids (C16:1 n7). As expected, the present study showed that alcohol feeding significantly reduced hepatic HNF4α expression, which was normalized by dietary MCT. In accordance, MCT/alcohol feeding up-regulated MTTP mRNA level and normalized ApoB mRNA level in the liver. Importantly, our cell culture study demonstrated polyunsaturated linoleic acid, rather than the monounsaturated oleic acid, inactivates HNF4α. We further demonstrated that knockdown of HNF4α in HepG2 cells reduced cellular ApoB100 and MTTP protein levels, leading to an increased cellular lipid accumulation and a reduced media ApoB100 level. Taken together, the present study suggests that unsaturated fatty acids may suppress, while MCT may normalize, VLDL secretion through modulating HNF4α activity.

Increasing evidence indicate that DNL may play an important role in regulating VLDL assembly and secretion (Park et al., 1997; Parks and Hellerstein, 2000). Blockage of fatty acid synthesis with the agent TOFA (5-tetradecyloxy-2-furoic acid), an inhibitor of acetyl CoA carboxylase, markedly inhibited the secretion of VLDL-triglyceride and ApoB, resulting in the decrease of plasma triglyceride in hamsters (Arbeeny et al., 1992). It is well known that hypercaloric high-carbohydrate/low fat diet, particularly rich in fructose, significantly increases plasma fasting and postprandial triglyceride level compared with high-fat, low-carbohydrate diet (Le et al., 2009; Parks and Hellerstein, 2000). Surplus carbohydrate intake stimulates hepatic DNL, which might either contribute directly to the VLDL-triglyceride fatty acid pool or indirectly alter the partitioning of fatty acids toward reesterification, resulting in increased VLDL-triglyceride secretion (Parks and Hellerstein, 2000; Schwarz et al., 2003). More recent findings indicate that *de novo* synthesized fatty acids or lipids can trigger several cellular signals and regulate metabolic homeostasis (Lodhi et al., 2011). The present study also demonstrated a possible link between DNL and VLDL secretion. First, dietary MCT up-regulated hepatic genes related to DNL and VLDL secretion. Second, dietary MCT normalized serum triglyceride level. Third, the *de novo* synthesized fatty acid, palmitic acid, in serum triglycerides were normalized by dietary MCT. Furthermore, hepatic DNL products such as palmitic acid thioester also regulate HNF4α activity. Further investigations are required to define the mechanistic link between hepatic DNL and VLDL secretion and the regulatory role of HNF4α.

In conclusion, this study demonstrated that chronic alcohol feeding with corn oil as fat source induced fatty liver in rats in association with increased hepatic unsaturated long chain fatty acids, decreased expression of VLDL secretion genes, and inactivation of HNF4α; All these alterations were attenuated by replacement of corn oil with MCT. HepG2 cell culture study demonstrated that polyunsaturated fatty acids, rather than monounsaturated fatty acid, inactivated HNF4α. Knockdown of HNF4α in HepG2 cells decreased hepatocyte proteins involved in VLDL secretion, leading to intracellular lipid accumulation. These results suggest that dietary medium chain triglyceride prevents alcoholic fatty liver, at least partially, through reducing polyunsaturated long chain fatty acids and preserving HNF4α.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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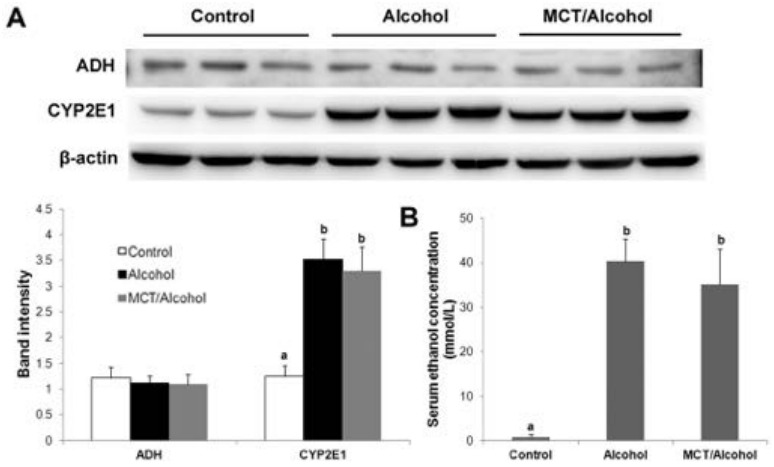

Figure 1.

Effects of alcohol and MCT on alcohol metabolism. A. Immunoblot analysis of hepatic ADH and CYP2E1 protein and band intensity of blots. B. Blood alcohol concentrations. Data are expressed as mean ± SD (n=3 in A; n=6-8 in B). Statistical differences were analyzed by ANOVA followed by Student-Newman-Keuls test, and means without a common letter differ at p<0.05.

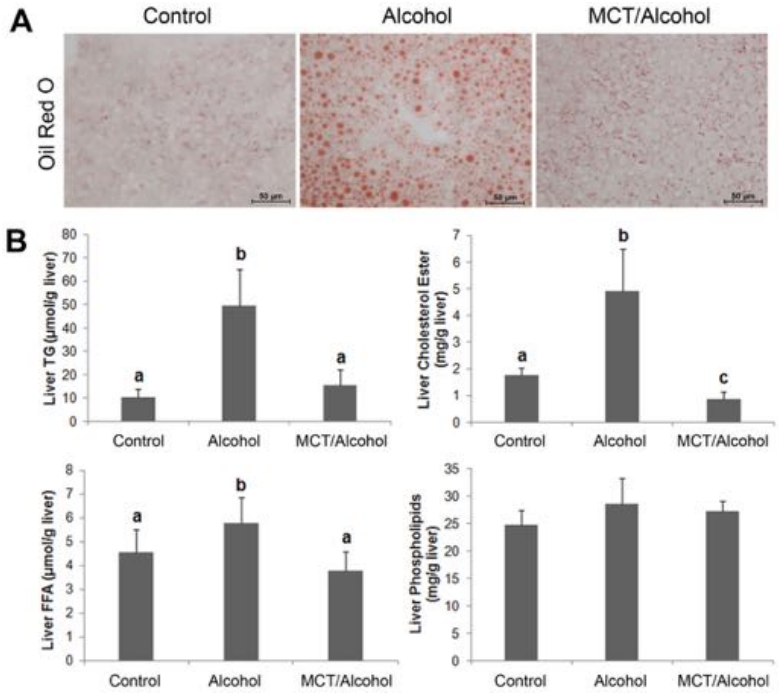

Figure 2.

Dietary MCT prevented hepatic lipid accumulation in rats exposed to alcohol for 8 weeks. (A) Oil red O staining of the neutral lipids in the liver. Lipid droplets are stained with red color. (B) Quantitative measurements of hepatic concentrations of triglyceride, cholesterol esters, FFA and phospholipids. Data are expressed as mean ± SD (n=6-8). Statistical differences were analyzed by ANOVA followed by Student-Newman-Keuls test, and means without a common letter differ at p<0.05.

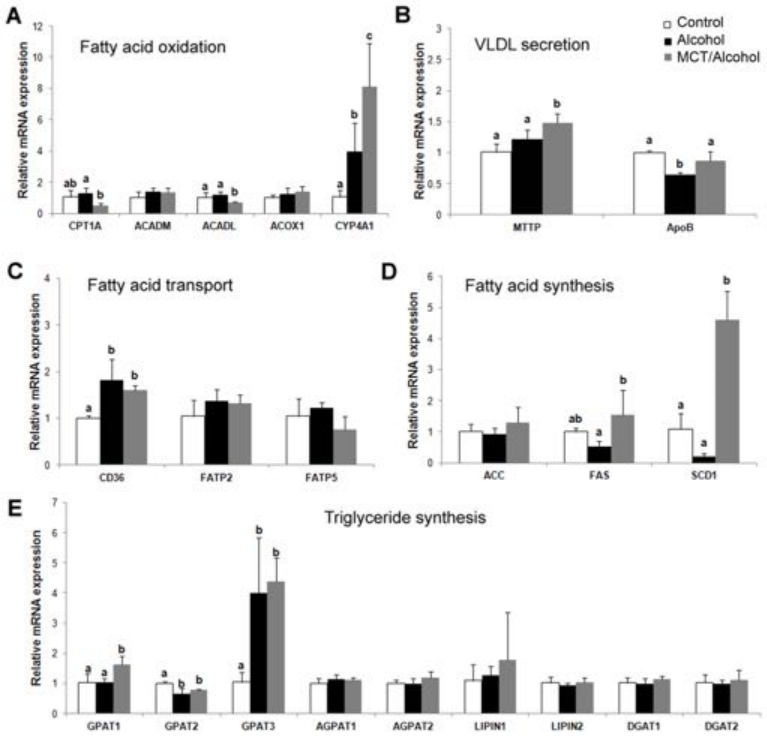

Figure 3.

Effects of dietary MCT on hepatic genes related to lipid metabolism in rats exposed to alcohol for 8 weeks. (A) Fatty acid oxidation genes. (B) VLDL secretion genes. (C) Fatty acid transport genes. (D) Fatty acid synthesis genes. (E) Triglyceride synthesis genes. Data are expressed as mean ± SD (n=6-8). Statistical differences were analyzed by ANOVA followed by Student-Newman-Keuls test, and means without a common letter differ at p<0.05.

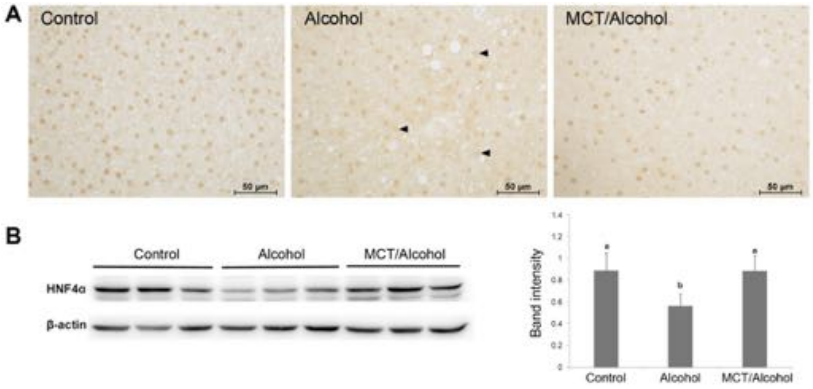

Figure 4.
Dietary MCT preserved HNF4α protein in the liver of rats exposed to alcohol for 8 weeks. (A) Immunohistochemical staining of HNF4α in liver sections. Arrows: nuclei with reduced staining. (B) Immunoblot analysis of liver HNF4α protein levels. Data are expressed as mean ± SD (n=6). Statistical differences were analyzed by ANOVA followed by Student-Newman-Keuls test, and means without a common letter differ at p<0.05.

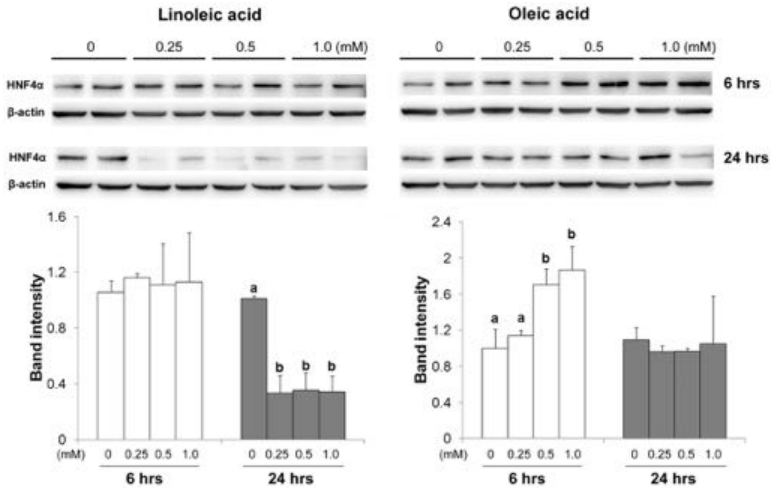

Figure 5.

Effects of unsayurated fatty acids on HNF4α in HepG2 cells. HepG2 cells were treated with linoleic acid or oleic acid at 0, 0.25, 0.5 or 1.0 mM for 6 or 24 hr. Immunoblot of HNF4α and band intensity of blots. Data are expressed as mean ± SD (n=4). Statistical differences were analyzed by ANOVA followed by Student-Newman-Keuls test, and means without a common letter differ at p<0.05.

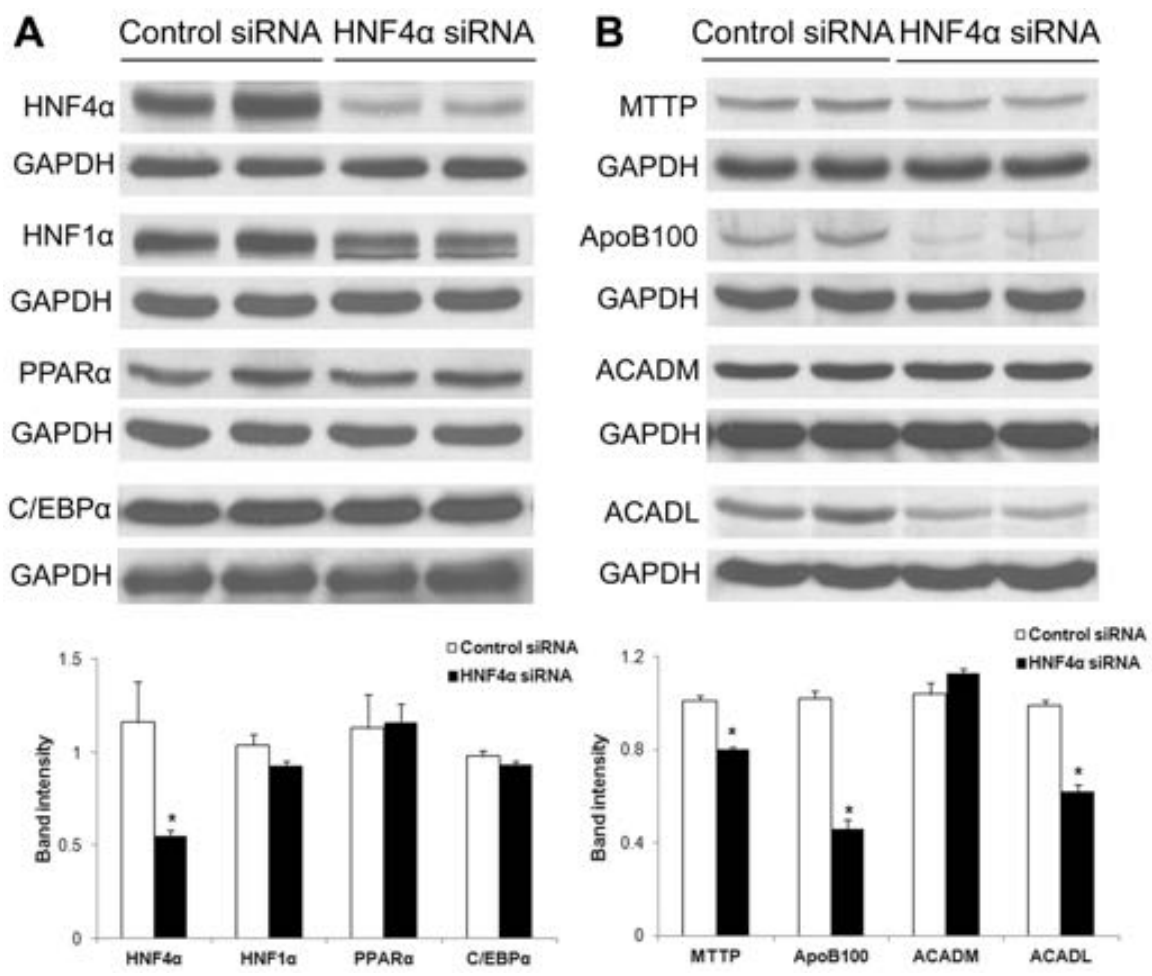

Figure 6.

Effects of HNF4α siRNA transfection on VLDL secretion related proteins in HepG2 cells. HepG2 cells were transfected with human HNF4α siRNA or control siRNA, and incubated for 3 days. (A) Immunoblot of HNF4α and band intensity of blots. B. Immunoblot of proteins related to VLDL secretion and fatty acid β-oxidation and band intensity of blots. Data are expressed as mean ± SD (n=4). Statistical differences were analyzed by independent samples t-test, and asterisks (*) indicate significant difference from the controls (p<0.05).

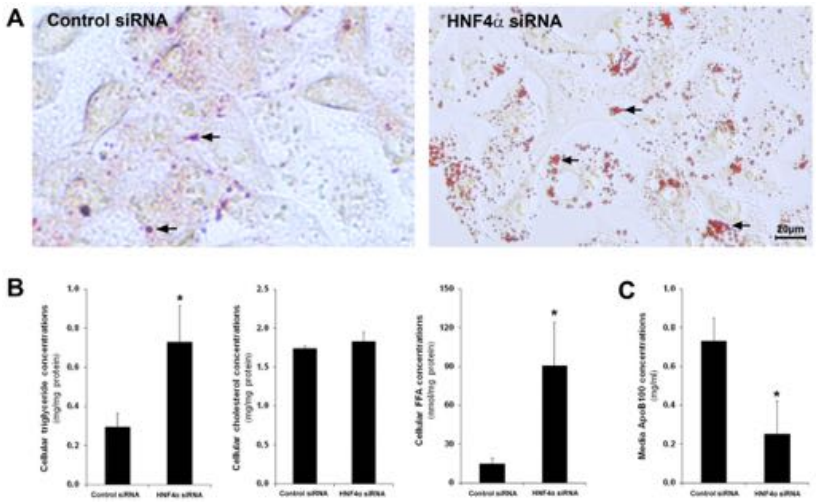

Figure 7.

Effects of HNF4α siRNA transfection on lipid accumulation and apoB100 secretion in HepG2 cells. HepG2 cells were transfected with human HNF4α siRNA or control siRNA, and incubated for 3 days. (A) Oil red O staining of neutral lipids in HepG2 cells. Arrows: lipid droplets. (B) Quantitative measurements of triglyceride, cholesterol and FFA in HepG2 cells. (C) Quantitative measurements of ApoB100 level in the medium. Data are expressed as mean ± SD (n=6 from 3 separate experiments). Statistical differences were analyzed by independent samples t-test, and asterisks (*) indicate significant difference from the controls (p<0.05).

Table 1

Composition of dietary calories of the control, alcohol and MCT/alcohol liquid diets

	Control	Alcohol	MCT/Alcohol
	(% of total calories)		
Protein	16	16	16
Carbohydrate	50	12	12
Corn oil	30	30	-
MCT	-	-	30
Safflower oil	4	4	4
Ethanol	-	38	38

MCT: Medium chain triglyceride.

Table 2

Body weight, liver weight, and blood parameters of rats fed liquid diets for 8 weeks

	Control	Alcohol	MCT/Alcohol
Body weight (g)	396.83 ± 31.02	399.12 ± 24.06	370.75 ± 23.37
Liver weight (g)	11.07 ± 1.26[a]	12.73 ± 1.08[b]	12.73 ± 0.99[b]
Blood glucose (mg/dL)	95.00 ± 15.29[a]	73.86 ± 9.37 [b]	74.63 ± 14.70 [b]
Serum β-hydroxybutyrate (mM)	5.51 ± 0.45[a]	15.25 ± 3.52 [b]	17.65 ± 4.21[b]

Rats were pair-fed liquid diets for 8 weeks. Data are means ± SD of 6-8 rats. Statistical differences were analyzed by ANOVA followed by Student-Newman-Keuls test, and means without a common letter differ at $p<0.05$.

Table 3

Fatty acid composition of hepatic total lipids in rats fed liquid diets for 8 weeks

Fatty acids (μg/g liver)	Control	Alcohol	MCT/Alcohol
C8:0	0.17 ± 0.12[a]	0.14 ± 0.07[a]	49.86 ± 8.49[b]
C10:0	0.57 ± 0.15[a]	0.69 ± 0.09[a]	193.05 ± 28.08[b]
C12:0	12.90 ± 0.93[a]	16.99 ± 0.97[a]	82.65 ± 10.43[b]
C14:0	240.66 ± 11.50[a]	337.76 ± 12.92[b]	416.10 ± 30.80[b]
C16:0	974.52 ± 52.47[a]	1781.29 ± 77.04[b]	1177.92 ± 82.55[a]
C16:1 n7	207.48 ± 20.20[a]	315.52 ± 24.57[b]	1195.87 ± 144.48[c]
C18:0	739.74 ± 29.25[a]	937.32 ± 30.41[b]	920.79 ± 35.99[b]
C18:1 n9	587.94 ± 42.82[a]	2365.07 ± 179.31[b]	771.96 ± 66.96[a]
C18:2 n6	1616.93 ± 152.97[a]	5320.43 ± 730.40[b]	906.13 ± 77.98[c]
C18:3 n3	20.23 ± 1.57[a]	76.46 ± 5.84[b]	12.51 ± 0.76[c]
C20:0	54.21 ± 3.83[a]	141.19 ± 9.03[b]	51.84 ± 4.82[a]
C20:1 n9	117.62 ± 8.66[a]	407.39 ± 35.11[b]	77.05 ± 6.65[c]
C20:2 n6	58.50 ± 5.14[a]	178.73 ± 13.54[b]	30.66 ± 2.66[c]
C20:3 n6	48.35 ± 1.60[a]	299.66 ± 47.72[b]	101.96 ±3.72[c]
C20:4 n6	1209.00 ± 64.30[a]	2394.09 ± 171.04[b]	1047.38 ± 63.22[a]
C20:5 n3	57.39 ± 5.50[a]	149.27 ± 12.54[b]	45.69 ± 5.05[a]
C22:0	42.24 ± 2.92[a]	47.23 ± 3.19[a]	33.05 ± 1.21[b]
C22:4 n6	77.64 ± 3.54[a]	377.87 ± 53.19[b]	62.82 ± 3.84[c]
C22:5 n6	48.56 ± 4.50[a]	205.92 ± 31.12[b]	98.84 ± 7.35[c]
C22:6 n3	375.96 ± 22.46[a]	655.08 ± 37.31[b]	542.01 ± 23.35[c]
C24:0	102.15 ± 5.32[ab]	114.74 ± 5.52[a]	92.79 ± 4.50[b]

Rats were pair-fed liquid diets for 8 weeks. Data are means ± SD of 6-8 rats. Statistical differences were analyzed by ANOVA followed by Student-Newman-Keuls test, and means without a common letter differ at $p<0.05$.

Table 4

Fatty acid composition of hepatic triglycerides in rats fed liquid diets for 8 weeks

Fatty acids (μg/g liver)	Control	Alcohol	MCT/Alcohol
C8:0	3.78 ± 1.15[a]	6.76 ± 1.04[a]	44.01 ± 10.76[b]
C10:0	1.82 ± 0.17[a]	3.03 ± 0.42[a]	131.72 ± 24.66[b]
C12:0	23.99 ± 1.09[a]	31.14 ± 1.81[a]	73.50 ± 13.25[b]
C14:0	79.75 ± 14.66[a]	261.11 ± 33.8[b]	325.87 ± 51.64[b]
C16:0	272.19 ± 38.26 [a]	1241.71 ± 138.72[b]	462.71 ± 79.04[a]
C16:1 n7	59.51 ± 21.42[a]	292.47 ± 46.56[b]	254.08 ± 98.12[b]
C18:0	39.25 ± 10.39[a]	136.77 ± 23.56[b]	82.01 ± 9.85[a]
C18:1 n9	131.19 ± 30.01[a]	976.71 ± 151.42[b]	152.04 ± 38.57[a]
C18:2 n6	145.95 ± 68.96[a]	1123.93 ± 276.54[b]	45.41 ± 13.96[a]
C18:3 n3	1.59 ± 0.99[a]	11.22 ± 3.50[b]	0.00 ± 0.00[a]
C20:0	9.26 ± 0.65[a]	38.75 ± 5.37[b]	16.51 ± 1.99[c]
C20:1 n9	13.56 ± 2.95[a]	60.65 ± 9.02[b]	27.11 ± 5.68[a]
C20:2 n6	1.91 ± 1.09[a]	26.62 ± 5.78[b]	0.00 ± 0.00[a]
C20:3 n6	6.84 ± 0.98[a]	73.59 ± 15.12[b]	4.27 ± 1.10[a]
C20:4 n6	20.76 ± 10.56[a]	178.72 ± 49.30[b]	4.89 ± 1.79[a]
C20:5 n3	11.53 ± 3.83[a]	38.54 ± 2.30[b]	14.44 ± 3.47[a]
C22:4 n6	4.88 ± 2.26[a]	61.55 ± 13.72[b]	0.51 ± 0.51[a]
C22:5 n6	1.14 ± 0.60[a]	30.55 ± 8.17[b]	1.01 ± 0.49[a]
C22:6 n3	0.00 ± 0.00[a]	20.73 ± 3.34[b]	0.00 ± 0.00[a]

Rats were pair-fed liquid diets for 8 weeks. Data are means ± SD of 6-8 rats. Statistical differences were analyzed by ANOVA followed by Student-Newman-Keuls test, and means without a common letter differ at $p<0.05$.

Table 5

Free fatty acid profile in the liver of rats fed liquid diets for 8 weeks

Fatty acids (μg/g live:r)	Control	Alcohol	MCT/Alcohol
C8:0	3.19 ± 1.20[a]	2.21 ± 0.53[a]	9.23 ± 1.44[b]
C10:0	3.59 ± 0.20[a]	3.64 ± 0.10[a]	18.03 ± 2.89[b]
C12:0	71.49 ± 2.77	77.06 ± 4.87	94.86 ± 11.55
C14:0	57.60 ± 17.97	68.11 ± 17.29	106.65 ± 23.48
C16:0	202.09 ± 16.96	206.47 ± 15.53	199.54 ± 18.89
C16:1 n7	26.90 ± 13.00	29.38 ± 4.32	25.40 ± 13.18
C18:0	92.37 ± 14.48	95.73 ± 13.78	117.64 ± 19.30
C18:1 n9	53.72 ± 11.92[a]	82.13 ± 7.63[b]	27.04 ± 3.88[c]
C18:2 n6	28.12 ± 6.94[a]	88.60 ± 26.17[b]	7.45 ± 0.81[c]
C20:0	6.49 ± 0.89	8.36 ± 0.87	7.70 ± 1.22
C20:4 n6	2.85 ± 0.78	6.23 ± 2.61	0.03 ± 0.03
C22:0	6.76 ± 0.60	6.69 ± 0.74	8.22 ± 0.97

Rats were pair-fed liquid diets for 8 weeks. Data are means ± SD of 6-8 rats. Statistical differences were analyzed by ANOVA followed by Student-Newman-Keuls test, and means without a common letter differ at $p<0.05$.

Table 6

Serum lipid concentration and fatty acid composition of serum triglycerides in rats fed liquid diets for 8 weeks

	Control	Alcohol	MCT/Alcohol
Serum triglyceride (mg/dL)	150.96 ± 20.63[a]	98.52 ± 13.04[b]	154.35 ± 44.27[a]
Serum free fatty acids (mM)	0.58 ± 0.14[a]	0.59 ± 0.24[a]	0.98 ± 0.41[b]
Serum cholesterol (mg/dL)	62.97 ± 4.37[a]	79.17 ± 8.34[b]	72.39 ± 16.06[ab]
Fatty acid profile of serum triglycerides (μg/ml)			
C8:0	1.34 ± 0.33[a]	0.26 ± 0.05[a]	17.07 ± 3.06[b]
C10:0	0.08 ± 0.02[a]	0.13 ± 0.04[a]	31.82 ± 6.45[b]
C12:0	1.75 ± 0.12[a]	1.82 ± 0.03[a]	8.22 ± 1.05[b]
C14:0	7.53 ± 0.25[a]	3.83 ± 0.42[b]	16.76 ± 2.20[c]
C16:0	16.36 ± 1.40[a]	9.00 ± 0.82[b]	21.37 ± 3.08[a]
C16:1 n7	4.84 ± 0.44[ab]	1.69 ± 0.29[a]	9.55 ± 2.72[b]
C18:0	5.28 ± 0.49[ab]	4.36 ± 0.25[a]	6.67 ± 0.65[b]
C18:1 n9	7.32 ± 0.76	6.12 ± 0.68	7.30 ± 1.25
C18:2 n6	7.25 ± 1.97[a]	4.42 ± 1.70[ab]	1.85 ± 0.42[b]
C20:0	1.11 ± 0.14	1.51 ± 0.18	1.69 ± 0.32
C20:3 n6	0.64 ± 0.03	0.62 ± 0.02	0.56 ± 0.01
C20:4 n6	0.99 ± 0.22	0.93 ± 0.35	0.39 ± 0.02
C22:0	0.16 ± 0.05	0.27 ± 0.04	0.50 ± 0.16

Rats were pair-fed liquid diets for 8 weeks. Data are means ± SD of 6-8 rats. Statistical differences were analyzed by ANOVA followed by Student-Newman-Keuls test, and means without a common letter differ at $p<0.05$.