Dietary saturated fatty acids reduce hepatic lipid accumulation but induce fibrotic change in alcohol-fed rats

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Background: In this study, we evaluated the influence of an ethanol-containing diet with high saturated fatty acids (SFAs) on alcoholic liver disease (ALD) in rats.

Methods: Male Wistar rats weighing about 160 g were divided into four groups: an ethanol (E) group fed an ethanol-containing liquid diet with 36% total calories as fat (corn oil, olive oil and safflower oil); a control (C) group pair-fed an isoenergetic diet without ethanol; an ethanol with saturated fat (EHS) group fed an ethanol-containing diet which contained 40% total calories as fat (90% lard); and a control with saturated fat (CHS) group fed an isoenergetic diet without ethanol, which contained 40% total calories as fat.

Results: After 8 weeks of treatment, the liver weight and plasma aspartate aminotransferase (AST) activities in E and EHS groups were significantly higher than those of C group. Significantly higher scores of inflammation, necrosis, and fatty changes were found in E group, whereas significantly higher scores of necrosis, bile duct hyperplasia, and fibrosis were found in EHS group. Although significantly lower plasma adiponectin concentrations were observed in both E and EHS groups, compared to C group, plasma adiponectin in EHS group was significantly higher than that in E group. There was no change in hepatic peroxisome proliferator activated receptor (PPAR)-α expression between E and C groups, and rats in EHS group showed a significantly elevated level compared to the other groups. A lower hepatic sirtuins (SIRT)-1 level was found in E group, but it did not reach statistical significance. Moreover, the highest plasma TGF-β1 level was found in EHS group. Compared to C group, the hepatic reduced glutathione/oxidized glutathione ratio and thiobarbituric acid (TBA)-reactive substance level were significantly increased in E and EHS groups; however, there was no significant difference between E and EHS groups. Significantly increased hepatic CYP2E1 expression was observed in both E and EHS groups, while at the same time, hepatic CYP2E1 in EHS group was the highest among all groups. The hepatic tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-10 concentrations in the E group were significantly higher than those in C group, whereas the hepatic IL-6 and IL-10 concentrations in ES group were significantly lower than those of E group.

Conclusions: These results suggested that dietary saturated fats may inhibit hepatic fat accumulation and induce hepatic fibrosis in rats under chronic alcohol intake.

Keywords: Alcoholic liver disease (ALD); saturated fatty acid (SFA); lard; rats

Introduction

Both dietary fatty acids and alcohol play important roles in the pathogenesis of alcoholic liver disease (ALD). The roles of different types of dietary fats in ALD are supported by epidemiologic studies. Those studies indicated that susceptibility to alcohol is related to different types of fatty acids in the diet. Nanji and French demonstrated that the expected mortality from cirrhosis was correlated with the dietary fatty acid composition, and saturated fatty acids (SFAs) were protective against ALD (1). Several animal studies also indicated that SFAs reduced hepatotoxicity induced by alcohol consumption including fatty liver, fibrotic changes, and necrosis (2-8). Recently, You *et al*. demonstrated that the protective effects of SFAs against alcoholic fatty liver may occur via the sirtuins (SIRT)-1-sterol regulatory element-binding protein 1 (SREBP-1) histone H3 axis which suppresses expressions of genes encoding lipogenic enzymes and slows the synthesis of hepatic fatty acids (9,10). However, most of these studies used vegetable oils such as palm oil, cocoa butter, and medium-chain triglycerides as fat sources for the SFAs. There is little evidence about animal SFAs on lipid synthesis and metabolism in ALD.

Lard is the most common source of dietary fat in Taiwan. Taiwanese often use lard to fry vegetables and make some Chinese-style desserts. In addition, mortality from ALD is increasing, and the public health topic of chronic alcohol consumption is becoming important in Taiwan. Therefore, we used lard as a source of dietary fats to investigate its effects on ALD in rats fed an ethanol-containing diet.

Materials and methods

Animals

Male Wistar rats (BioLasco Taiwan, Taiwan) weighing about 160 g were used in this experiment. All rats were housed in individual stainless-steel cages in an animal room maintained at 22±2 ℃ with 50-70% humidity and a 12-h light-dark cycle. All rats were allowed free access to a standard rodent diet (LabDiet 5001 Rodent Diet; PMI Nutrition International, MO, USA) and water for 1 week during acclimation before the study. All procedures were approved by the Institutional Animal Care and Use Committee of Taipei Medical University.

Study protocol

After 1 week of acclimation, rats were assigned to groups based on their levels of activity of plasma aspartate transaminase (AST) and alanine transaminase (ALT), so there would be no significant differences between groups in their plasma AST and ALT activity levels. The four groups were as follows: an ethanol (E) group fed an ethanol liquid diet (containing 36% energy as fat) with a Ritcher drinking tube; a control (C) group pair-fed an isoenergetic diet without ethanol; an ethanol with high-SFA diet (EHS) group fed an ethanol-containing diet, which contained 90% lard and 10% soybean oil substitute for corn oil, olive oil and safflower oil in the diet of E group; and a control with high-fat diet (CHS) group fed an isoenergetic diet without ethanol, which contained lard and soybean oil as the fat source. Compositions of the experimental diets of all groups are given in *Table 1*. After 8 weeks of the experimental period, rats were anesthetized and sacrificed. Blood samples were collected in heparin-containing tubes and centrifuged (1,200 ×g for 15 min at 4 ℃) to separate the plasma. All plasma samples were stored at –80 ℃ until being assayed. Liver tissues were rapidly excised and stored at –80 ℃ for further analysis.

Measurements and analytical procedures

Liver function
Plasma AST and ALT activities were measured as indices of liver function using a colorimetrical method with an automatic analyzer, Beckman Synchron LXTM system (GMI, MN, USA). The system monitors the change in absorbance at 340 nm, and then calculates and expresses the AST and ALT activities.

Hepatic histopathology and plasma TGF-β1 concentration
Formalin-fixed liver tissues were stained with hematoxylin-eosin (H&E) and trichrome stains. A semiquantitative histological evaluation was carried out by a pathologist blinded to the treatment groups to assess the degree of tissue inflammation, fatty changes, necrosis of hepatocytes, and tumor burden. The grading ranged 0-4 where 0= absent, 1= trace, 2= mild, 3= moderate, and 4= severe.

The plasma TGF-β1 concentration was measured using a rat TGF-β1 kit (R&D Systems, MN, USA), and the optical density was read at 450 nm using a microplate reader (Molecular Devices, CA, USA).

Factors influencing lipogenesis and lipolysis
Plasma adiponectin concentration
The plasma adiponectin concentration was measured using an enzyme-linked immunosorbent assay (ELISA)

Table 1 Composition of the experimental diet in each group*

Ingredient#	Concentration [g/L (1,000 kcal)]			
	C	E	CHS	EHS
Casein	41.4	41.4	41.4	41.4
L-cysteine	0.5	0.5	0.5	0.5
DL-methionine	0.3	0.3	0.3	0.3
Corn oil	8.5	8.5	–	–
Olive oil	28.4	28.4	–	–
Safflower oil	2.7	2.7	–	–
Lard	–	–	40.0	40.0
Soybean oil	–	–	4.4	4.4
Choline bitartrate	0.53	0.53	0.53	0.53
Fiber	10	10	10	10
Xanthan gum	4	4	4	4
ICN: AIN-76 vitamins	2.5	2.5	2.5	2.5
ICN: AIN-76 minerals	2.6	2.6	2.6	2.6
Maltodextrin	115.2	25.6	104.2	14.6
Ethanol	–	50	–	50

*, C, control group; CHS, group fed the control diet high in saturated fatty acids; E, ethanol group; EHS, group fed the ethanol-containing diet high in saturated fatty acids. #, L-cysteine, DL-methionine, choline bitartrate, fiber, AIN-76 vitamins, AIN-76 minerals, and maltodextrin were purchased from ICN Biochemicals (Costa Mesa, CA, USA). Xanthan gum, ethanol, and glutamine were purchased from Sigma-Aldrich (St Louis, MO, USA). Corn oil and olive oil were purchased from God Bene Enterprise (Yunlin, Taiwan). Safflower oil was purchased from Taiwan Sugar Corporation (Taipei, Taiwan).

kit (AssayMax rat adiponectin ELISA kit, MO, USA). The optical density was read at 450 nm for all cytokines using a microplate reader (Molecular Devices, CA, USA).

Hepatic SIRT-1 expression

A Western blot analysis was performed to determine the expression of SIRT-1. Liver tissues were homogenized in 5 volumes of buffer (25 mM HEPES at pH 7.6, 300 mM NaCl, 1.5 mM MgCl₂, 1 mM EDTA, 1% Triton-X, and 10% glycerol). After shaking for 10 min, the homogenate was centrifuged at 13,200 ×g for 40 min at 4 ℃. The supernatant was centrifuged at 55,000 rpm for 60 min at 4 ℃. The supernatant was separated by 10% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then proteins were electroblotted onto a polyvinylidene difluoride (PVDF) transfer membrane

(Amersham Biosciences, UK). These blots were incubated with rabbit monoclonal anti-rat SIRT-1 (Aviva Systems Biology, CA, USA) and mouse anti-actin monoclonal antibodies (Chemicon International, CA, USA). Finally, the blot was treated with goat anti-rabbit immunoglobulin G (IgG)-horseradish peroxidase (HRP) (Chemicon International, CA, USA), and specific bindings of antibodies were detected using a Western Lightning kit (PerkinElmer Lifesciences, MA, USA). Bands were quantified using Image-Pro Plus software (vers. 4.5.0.29; MD, USA).

Plasma non-esterified fatty acid (NEFA) levels

Plasma NEFAs were determined using a commercial kit (Randox Laboratories, UK). Ten microliters of sample or standard was added to 200 µL of reagent 1 (0.04 mol/L phosphate buffer, 3 mmol/L magnesium chloride, 0.3 U/mL acyl coenzyme A synthetase, 1.5 U/mL ascorbate oxidase, 0.9 mmol/L coenzyme A, 5.0 mmol/L ATP, and 1.5 mmol/L 4-aminoantipyrine), mixed well, and incubated for 5 min at 37 ℃. Then, 400 µL of reagent 2 (0.3% w/v phenoxyethanol, 10.6 mmol/L maleimide, 10 U/mL acyl coenzyme A oxidase, 7.5 U/mL peroxidase, and 1.2 mmol/L TOOS) was added. After incubating the mixture at 37 ℃ for 5 min, NEFA concentrations were measured at 550 nm.

Hepatic peroxisome proliferator activated receptor (PPAR)-α expression

A Western blot analysis was performed to determine the expression of PPAR-α. Liver tissues (0.5 g) were homogenized in 1.5 mL RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, and 1% NP-40 at pH 7.5). After an ice bath for 30 min, the homogenate was centrifuged at 10,000 ×g for 10 min at 4 ℃. The supernatant was separated by 10% SDS-PAGE. Then proteins were electroblotted onto a PVDF transfer membrane (Amersham Biosciences, UK). These blots were incubated with rabbit monoclonal anti-rat PPAR-α (Aviva Systems Biology, CA, USA) and mouse anti-actin monoclonal antibodies (Chemicon International, CA, USA). Finally, the blot was treated with goat anti-rabbit IgG-HRP (Chemicon International, CA, USA), and specific bindings of antibodies were detected using a Western Lightning kit (PerkinElmer Lifesciences, MA, USA). Bands were quantified using Image-Pro Plus software (vers. 4.5.0.29, MD, USA).

Oxidative stress

Hepatic reduced glutathione (GSH)/oxidized glutathione (GSSG) ratio

Liver tissue (0.5 g) was homogenized in 1.5 mL ice cold buffer (50 mM Tris at pH 7.2, 150 mM NaCl, and

1% Triton-X) plus 0.1% of a protease inhibitor. The homogenate was then shaken on ice for 90 min. After shaking, the homogenates were centrifuged at 3,000 ×g and 4 ℃ for 15 min. The GSH/GSSG ratio, lipid peroxide concentration, and tumor necrosis factor-α (TNF-α) concentration of the supernatants were analyzed.

The GSH concentration was measured spectrophotometrically at 405 nm according to the method of Tietze (11), and the GSSG concentration was measured spectrophotometrically at 405 nm according to the method of Griffith (12). The ratio of GSH/GSSG was calculated as (GSH-2GSSG)/(GSSG).

Plasma and hepatic thiobarbituric acid (TBA)-reactive substances (TBARSs)

Lipid peroxidation was determined by measuring the concentration of TBARSs in the liver with minor modifications (13). Briefly, 20 mL of liver homogenates or plasma sample or 1,1,3,3-tetramethoxypropane (as the standard) was shaken with 800 mL of 0.22% H_2SO_4 in a 2-mL centrifuge tube. Then, 100 mL of 10% phosphotungstic acid and 200 mL of 0.67% TBA were added to the mixture, mixed well, and warmed for 60 min in a boiling water bath followed by rapid cooling. This was shaken into 600 mL of an n-butanol layer in a separation tube, and the n-butanol layer was measured fluorometrically (at respective excitation and emission wavelengths of 531 and 590 nm) using a Wallac Victor-2 1420 Multilabel Counter (PerkinElmer, MA, USA).

Hepatic microsomal CYP2E1 protein expression

Liver tissues were homogenized in 10 volumes of ice-cold buffer (0.25 M sucrose, 10 mM Tris-HCl, and 0.25 mM phenylmethylsulfonyl fluoride at pH 7.4). The homogenates were centrifuged at 17,000 ×g for 20 min at 4 ℃. The supernatant was transferred to a new tube and centrifuged at 105,000 ×g for 60 min at 4 ℃ to separate the microsomes. The microsomal pellet was dissolved in 50 mM potassium phosphate buffer containing 1 mM EDTA and 1 mM DTT (pH 7.4). Microsomal proteins (30 μg) were separated by 10% SDS-PAGE. After separation, the proteins were electroblotted onto a PVDF transfer membrane (Amersham Biosciences, UK). These blots were then incubated with mouse monoclonal anti-rat CYP2E1 (Oxford Biomedical Research, MI, USA) and mouse anti-actin monoclonal (Chemicon International, CA, USA) antibodies. Finally, the blot was treated with goat anti-mouse IgG-HRP (Chemicon International, CA, USA) or goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, CA, USA), and specific bindings of antibodies were detected using a Western Lightning kit (PerkinElmer Lifesciences, MA, USA). The bands were quantified using Image-Pro Plus 4.5 software analysis (vers. 4.5.0.29, MD, USA).

Inflammatory factors

Hepatic cytokine concentrations were measured using ELISA kits. TNF-α, interleukin (IL)-1β, IL-6, and IL-10 concentrations were respectively determined with a rat TNF-α/TNFSF1A kit, a rat IL-1β/IL-1F2 kit, a rat IL-6 kit, and a rat IL-10 kit (all from R&D Systems, MN, USA). The optical density was read at 450 nm for all cytokines using a microplate reader (Molecular Devices, CA, USA).

Statistical analysis

All data are expressed as the mean ± standard deviation (SD). A one-way analysis of variance (ANOVA) followed by Fisher's test was used to determine statistical differences among groups using SAS software vers. 8.0 (SAS Institute, Cary NC, USA). Statistical significance was assigned at the P<0.05 level.

Results

Energy and alcohol intake

The average energy intake in the four groups was as follows: C group (74.8±11.6 kcal/day), CHS group (75.1±10.6 kcal/day), E group (76.4±9.6 kcal/day), and EHS group (63.9±9.5 kcal/day). There were no differences among these groups in average energy intake. The average alcohol intake levels in the groups consuming ethanol were as follows: E group (3.8±0.5 g/day), and EHS group (3.2±0.5 g/day). No difference was observed between E and EHS groups in alcohol intake.

Body weight (BW) and relative liver weight

The initial BW, final BW, and relative liver weight of rats in each group are listed in *Table 2*. There were no significant differences in initial BWs of rats among all groups, however, EHS group showed the lowest BW among all groups. In addition, the relative liver weights of E and EHS groups were significantly higher than that of C group (P<0.05).

Liver function

Plasma AST and ALT activities of each group are shown in

Table 2 Initial body weight, final body, liver weight, and relative liver weight of rats in each group*

Group[#]	Initial body weight (g)	Final body weight (g)	Liver weight (g)	Relative liver weight[&] (%)
C	215.6±6.4[a]	403.5±4.3[b]	9.8±0.2[a,b]	2.4±0.0[a]
CHS	232.1±10.0[a]	401.8±6.9[b]	9.1±0.2[a]	2.3±0.0[a]
E	223.9±3.9[a]	396.1±5.0[b]	12.1±0.3[c]	3.0±0.1[b]
EHS	207.9±8.3[a]	327.3±10.8[a]	10.7±0.5[b]	3.2±0.1[b]

*, values are expressed as the mean ± SEM (n=8). Means in the same column with different superscript letters significantly differ (P<0.05); [#], C, control group; CHS, group fed the control diet high in saturated fatty acids; E, ethanol group; EHS, group fed the ethanol-containing diet high in saturated fatty acids; [&], relative liver weight: (liver weight/body weight) ×100%.

Table 3 Plasma aspartate transaminase (AST) and alanine transaminase (ALT) activities of rats in each group*

Group[#]	AST activity (U/L)	ALT activity (U/L)
C	83.8±2.7[a]	50.4±4.1[a]
CHS	83.9±3.1[a]	46.0±3.2[a]
E	199.5±17.7[b]	90.4±12.0[a,b]
EHS	231.5±60.8[b]	185.5±68.6[b]

*, values are expressed as the mean ± SEM (n=8). Means in the same column with different superscript letters significantly differ (P<0.05); [#], C, control group; CHS, group fed the control diet high in saturated fatty acids; E, ethanol group; EHS, group fed the ethanol-containing diet high in saturated fatty acids.

Table 4 Hepatic histopathology scores of rats in each group*

| Group[#] | Inflammatory cell infiltration | Degeneration and necrosis | Fatty change | | | Hypertrophy of hepatocytes | Bile duct hyperplasia | Fibrosis |
			Macrovesicular	Microvesicular	Midzonal and portal area			
C	1.6±0.2[a]	1.4±0.2[a]	1.6±0.2[a]	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	1.4±0.2[a,b]	0.8±0.4[a]
CHS	2.0±0.0[a]	1.6±0.2[a]	1.8±0.2[a]	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	1.8±0.2[b,c]	1.6±0.2[a,b]
E	2.8±0.2[b]	3.0±0.0[b]	2.8±0.2[b]	1.8±0.2[b]	0.0±0.0[a]	0.0±0.0[a]	1.0±0.0[a]	1.0±0.3[a]
EHS	1.8±0.2[a]	3.0±0.0[b]	1.4±0.2[a]	0.0±0.0[a]	0.4±0.4[a]	2.6±0.2[b]	2.0±0.0[c]	2.0±0.0[b]

*, values are expressed as the mean ± SEM (n=8). Means in the same column with different superscript letters significantly differ (P<0.05); [#], C, control group; CHS, group fed the control diet high in saturated fatty acids; E, ethanol group; EHS, group fed the ethanol-containing diet high in saturated fatty acids.

Table 3. The plasma AST activities of E and EHS groups were significantly higher than that of C group after the 8-week feeding (P<0.05). The plasma ALT activity of E group showed an increasing trend compared to C group. Otherwise, the plasma ALT activity of EHS group was significantly higher than those of C and CHS groups (P<0.05).

Hepatic histopathology and plasma TGF-β1 concentration

Hepatic histopathology scores are presented in *Table 4*.

As shown in *Table 4*, inflammatory cell infiltration, cell degeneration and necrosis, and fatty changes were observed in E group. On the other hand, inflammatory cell infiltration and fatty changes in EHS group were lower than those in E group; however, significantly higher hypertrophy of hepatocytes, bile duct hyperplasia, and fibrosis were only found in EHS group (P<0.05). As shown in *Figure 1A*, fatty changes were found in E group; in addition, collagen generation was observed in CHS, E, and EHS groups (*Figure 1B*). Plasma TGF-β1 concentrations in the 8th week of the experiment are given in *Table 5*. A significantly higher
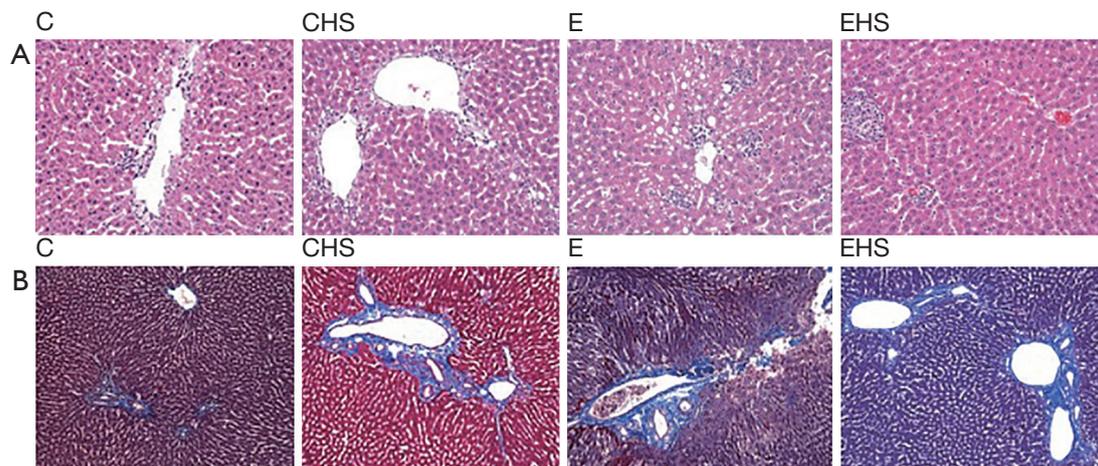

Figure 1 Representative photomicrographs of livers. (A) H&E stain (magnification: ×200); (B) trichrome stain (magnification: ×100). C, control group; CHS, group fed the control diet high in saturated fatty acids; E, ethanol group; EHS, group fed the ethanol-containing diet high in saturated fatty acids.

Table 5 Plasma transforming growth factor (TGF)-β1 of rats in each group*

Group[#]	TGF-β1 (ng/mL)
C	9.5±0.9[a]
CHS	22.1±8.3[a,b]
E	7.9±1.9[a]
EHS	35.0±6.8[b]

*, values are expressed as the mean ± SEM (n=8). Means in the same column with different superscript letters significantly differ (P<0.05); [#], C, control group; CHS, group fed the control diet high in saturated fatty acids; E, ethanol group; EHS, group fed the ethanol-containing diet high in saturated fatty acids.

Table 6 Plasma adiponectin and free fatty acid levels of rats in each group*

Group[#]	Adiponectin (μg/mL)	Free fatty acids (mmol/L)
C	15.7±0.8[c]	0.57±0.04[b]
CHS	14.6±0.5[c]	0.46±0.04[a,b]
E	8.2±0.6[a]	0.39±0.04[a]
EHS	11.6±1.3[b]	0.39±0.07[a]

*, values are expressed as the mean ± SEM (n=8). Means in the same column with different superscript letters significantly differ (P<0.05); [#], C, control group; CHS, group fed the control diet high in saturated fatty acids; E, ethanol group; EHS, group fed the ethanol-containing diet high in saturated fatty acids.

plasma TGF-β1 concentration was observed in EHS group (P<0.05).

Factors influencing lipogenesis and lipolysis

As shown in *Table 6*, a significantly lowest plasma adiponectin concentration was found in the E group (P<0.05). Plasma adiponectin in EHS group was lower than that in C group, but higher than that in E group (P<0.05). The plasma free fatty acid levels of each group are given in *Table 6*. It indicated that plasma free fatty acid levels in E and EHS groups were significantly lower than that of C group (P<0.05). As shown in *Figure 2*, lower hepatic SIRT-1 expression was found in E group, but it failed to reach

statistical significance. Moreover, there was no significant difference in hepatic SIRT-1 expression between E and EHS groups. As shown in *Figure 2*, hepatic PPAR-α in EHS group was significantly higher than those of the other three groups (P<0.05).

Oxidative stress

Biomarkers of oxidative stress of rats in each group are presented in *Table 7*. E group showed a significantly lower hepatic GSH/GSSG ratio (P<0.05); however, the hepatic GSH/GSSG ratio between E and EHS groups showed no significant difference. Hepatic TBARS concentrations in E and EHS groups were significantly higher than that in C
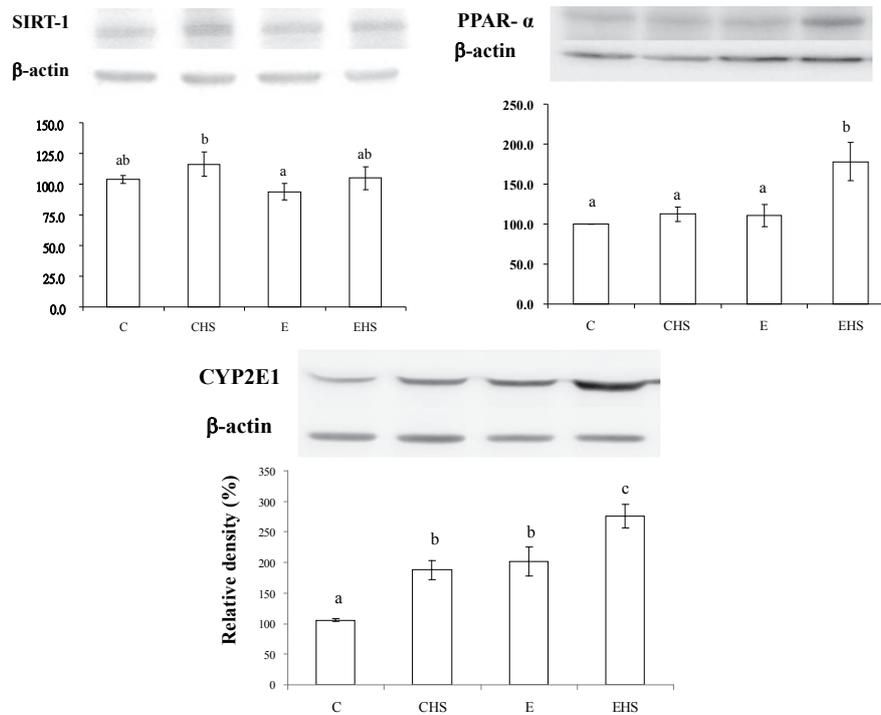
Figure 2 Hepatic sirtuins (SIRT)-1, peroxisome proliferator activated receptor (PPAR)-α and CYP2E1 expressions of rats in each group. C, control group; CHS, group fed the control diet high in saturated fatty acids; E, ethanol group; EHS, group fed the ethanol-containing diet high in saturated fatty acids. Bars with different letters significantly differ at the P<0.05 level according to Fisher's post-hoc test.

Table 7 Biomarkers of oxidative stress of rats in each group*		
Group[#]	GSH/GSSG	TBARSs (μM)
C	13.3±1.7[b]	73.1±4.3[a]
CHS	13.3±1.5[b]	81.6±3.9[a]
E	8.2±1.2[a]	101.7±3.2[b]
EHS	9.7±1.6[a,b]	96.9±5.3[b]

*, values are expressed as the mean ± SEM (n=8). Means in the same column with different superscript letters significantly differ (P<0.05); [#], C, control group; CHS, group fed the control diet high in saturated fatty acids; E, ethanol group; EHS, group fed the ethanol-containing diet high in saturated fatty acids. GSH, reduced glutathione; GSSG, oxidized glutathione; TBARSs, thiobarbituric acid-reactive substances.

group (P<0.05). Moreover, CYP2E1 expression is shown in *Figure 2*. CYP2E1 expression of E group was significantly higher than that of C group (P<0.05). In addition, EHS group showed the highest CYP 2E1 expression among all groups (P<0.05).

Inflammatory factors

Hepatic cytokines in each group are given in *Table 8*. E group showed significantly higher hepatic TNF-α, IL-1β, IL-6, and IL-10 concentrations than those of rats in C group. Hepatic TNF-α and IL-1β concentrations in EHS group did not significantly differ compared to those of E group; however, hepatic IL-6 and IL-10 in EHS group were significantly lower than those of E group (P<0.05).

Discussion

In this study, we used lard as a source of dietary fat to investigate its effect on ALD in rats fed an ethanol-containing diet for 8 weeks.

BW, liver weight, and alcohol intake

The average energy intake was similar in all groups. The alcohol intake volume also showed no difference in the alcohol-fed groups. However, significantly higher relative liver weights in E and EHS groups indicated that hepatomegaly

Table 8 Hepatic tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-10 levels of rats in each group*

Group[#]	TNF-α (pg/mg liver)	IL-1β (pg/mg liver)	IL-6 (pg/mg liver)	IL-10 (pg/mg liver)
C	60.8±4.8[a]	59.7±3.0[a]	93.9±4.2[a,b]	90.2±3.3[a]
CHS	76.6±3.2[a,b]	65.5±2.8[a,b]	85.7±3.8[a]	95.4±5.3[a]
E	86.8±4.2[c]	77.4±1.6[c]	124.3±3.1[c]	113.8±5.1[b]
EHS	74.2±5.0[b,c]	68.9±4.0[b,c]	104.5±6.1[b]	89.3±3.2[a]

*, values are expressed as the mean ± SEM (n=8). Means in the same column with different superscript letters significantly differ (P<0.05); [#], C, control group; CHS, group fed the control diet high in saturated fatty acids; E, ethanol group; EHS, group fed the ethanol-containing diet high in saturated fatty acids.

had occurred in both groups after 8 weeks of alcohol exposure (*Table 2*). Alcohol intake was reported to increase the relative liver weight in rats (14). Moreover, a previous study showed that isocaloric substitution of carbohydrates by ethanol resulted in significantly lower weight gain but a higher relative liver weight despite similar energy intake levels (15). Our findings indicate that alcohol intake accompanied with high dietary SFAs produced the lowest final BW even under similar energy consumption, but showed no effect on hepatomegaly induced by chronic alcohol feeding.

Liver function and hepatic histopathology

AST and ALT activities are markers of liver damage as opposed to alcohol misuse (16). Both transaminases are found in hepatocytes, but AST is also found in skeletal and myocardial cells. In alcohol-related liver damage, AST is elevated more than ALT, at least in part as a reflection of alcohol-related skeletal damage (17). Therefore, to evaluate liver damage caused by chronic ethanol feeding, plasma AST and ALT activities were determined in the present study. Increased plasma AST and ALT activities in rats fed an ethanol-containing diet after the 8-week feeding revealed liver injury in both groups (*Table 3*). In E group, inflammatory cell infiltration and fat accumulation were observed from results of the histopathology examinations (*Table 4*). However, rats fed alcohol and high saturated fats had slight fatty changes and inflammation but significantly severe hypertrophy of hepatocytes and fibrosis in the liver (*Table 4*). These results indicated that liver injury was present in E and EHS groups, and the type of liver injury differed between the E and EHS groups.

Factors influencing lipogenesis and lipolysis

Alcohol is thought to cause fatty liver by altering the

hepatic NADH/NAD+ ratio, that is, inhibiting fatty acid oxidation and stimulating lipogenesis (18,19). More recent studies indicated that alcohol intake may influence the activity of PPAR-α, SREBP-1, and AMP-dependent protein kinase (AMPK) directly or by way of effects on adiponectin (20-23). Adiponectin, a hormone largely secreted by adipocytes, has two receptors, receptors 1 (AdipoR1) and 2 (AdipoR2). AdipoR1 is expressed in various tissues with a relatively high level in skeletal muscle, whereas AdipoR2 is predominantly expressed in the liver. Adiponectin protects against ALD by decreasing inflammation and lipogenesis; however, previous studies showed that adiponectin was lower in ethanol-fed rats and mice (24,25). You *et al*. also indicated that inhibition of SIRT-1 activity was associated with an increase in the acetylated active nuclear form of SREBP-1c in livers of ethanol-fed mice (21). Moreover, activated PPAR-α induces the expression of genes involved in mitochondrial and peroxisomal free fatty acid β-oxidation. PPAR-α also inhibits the lipogenic pathway by inducing malonyl CoA, a precursor of fatty acid synthesis (26). *In vivo* experiments on ethanol-fed rodents reported animal-specific differences in the effects of ethanol on hepatic PPAR-α protein levels. In SV/129 mice and in rats, ethanol administration decreased PPAR-α protein levels (27). On the other hand, PPAR-α protein did not change in ethanol-fed C57BL/6J mice; however PPAR-α binding to DNA was significantly impaired and some PPAR-α target genes were downregulated (28). However, we found that the plasma adiponectin level decreased, but SIRT-1 and PPAR-α expressions exhibited no changes in E group in this study (*Figure 2*). Although hepatic lipid accumulation was observed in rats fed ethanol, upstream factors related to lipogenesis and lipolysis, such as SIRT-1 and PPAR-α expressions, were not transformed in this study. Because it has been shown previously that chronic alcohol intake affected SIRT-1 activity (21), not protein itself, we should

confirm the activities of these factors in a future study.

On the other hand, the increased expressions of adiponectin and PPAR-α in EHS group indicated that high dietary SFAs improved the β-oxidation of fatty acids in the liver via upregulation of the adiponectin-PPAR-α pathway, and then inhibited hepatic accumulation induced by chronic alcohol intake (*Table 6*, *Figure 2*). Our results are similar to those of a previous study which showed that a high-saturated-fat diet increased β-oxidation of fatty acids in the liver against alcoholic fatty changes (29). We also found that plasma free fatty acids decreased in both groups E and ES (*Table 6*), so we supposed the lower lipogenic response induced by SFAs occurred in liver tissues, not in adipose tissues.

In addition, PPAR-α also regulates a number of other biological processes such as cell proliferation, apoptosis, inflammation, oxidative stress, and hepatocarcinogenesis (30,31). In our study, EHS group showed significantly higher hepatic PPAR-α protein expression than did the other three groups (*Figure 2*), and we suggest that the higher hepatic PPAR-α protein expression in EHS group may have contributed to fewer fatty changes; however, it may also indicate possible hepatocarcinogenesis in this group.

Hepatic fibrosis

We also observed a higher plasma TGF-β1 level in the EHS group in our study. This result matches results of the hepatic histological analysis (*Table 4*, *Figure 1*). Carmiel-Haggai *et al*. showed that high saturated fat may cause hepatic fibrosis via increasing TGF-β (32). In addition to fibrosis, TGF-β also regulates chronic liver disease by contributing to all stages of disease progression from initial liver injury through inflammation and fibrosis to cirrhosis and hepatocellular carcinoma (33). Our data showed that chronic ethanol feeding together with a high-saturated-fat diet induced significantly higher plasma TGF-β concentrations; this indicated that hepatocellular carcinoma may develop in this group.

Oxidative stress

Oxidative stress is one of the mechanisms of alcoholic liver injury. As shown in *Table 7*, oxidative stress was found in rats after 8 weeks of alcohol exposure (group E); however, combining ethanol with saturated fats did not alter the hepatic GSH/GSSG ratio or TBARS concentration. This indicates that saturated fats, especially lard, did not change

oxidative stress at least in this ethanol-exposure animal model. On the other hand, overexpression of hepatic CYP2E1 leads to ROS generation and highly reactive free radicals including superoxide anions and hydroxyl radicals which then result in oxidative stress in alcoholic liver injury (34,35). As shown in *Figure 2*, hepatic CYP2E1 expression increased in E group. Surprisingly, regardless of whether ingesting alcohol or not, high dietary SFAs increased hepatic CYP2E1 expression, and the highest level was observed in EHS group. Cytochrome P450 2E1 is involved in the microsomal ethanol-oxidizing system and is induced by heavy ethanol use. Morgan *et al*. stated that CYP2E1 can contribute to the development of hepatocarcinogenesis in two ways (36). First, CYP2E1 produces ROS that can lead to cell death and/or DNA damage and cancer initiation (37). Second, CYP2E1 oxidizes xenobiotics, including the carcinogens dimethylnitrosamine, aflatoxin, vinyl chloride, and dimethylhydrazine (38). Thus, even though oxidative stress, including lipid peroxidation and antioxidative substrate levels, was not increased by the high dietary SFA intake, CPY2E1 overexpression induced by the high dietary SFA intake still is a risk factor for hepatocarcinogenesis.

Proinflammatory and anti-inflammatory cytokines

Alcohol consumption can promote bacterial overgrowth, which may cause the accumulation of endotoxin. In addition, alcohol-induced gut barrier disruption leads to increased intestinal permeability, and excess amounts of endotoxin may transfer from the intestines to the liver and the general circulation and contribute to an inflammatory response (39,40). An increasing circulating endotoxin level induced by chronic alcohol intake activates innate immunity via stimulation of Kupffer cells to produce proinflammatory cytokines, such as TNF-α and IL-1β, that cause liver injury (41-44). In the present study, we observed that TNF-α and IL-1β levels significantly increased in E group compared to C group (*Table 8*). These results are consistent with those of previous studies (45-48). On the other hand, compared to E group, TNF-α and IL-1β levels in EHS group did not change. Thus, these results suggest that alcohol intake accompanied by high SFAs did not improve the production of proinflammatory cytokines.

In addition, activation of innate immunity also stimulates Kupffer cells to produce hepatoprotective cytokines (e.g., IL-6) and anti-inflammatory cytokines (e.g., IL-10 and adiponectin) in ALD (49,50). Recent studies suggested that both IL-6 and IL-10 play compensatory roles in protecting

against ALD. Elevated IL-6 stimulates STAT3 activation in hepatocytes and upregulates the expression of antiapoptotic genes (e.g., Bcl-2 and Bcl-xL), antioxidative genes (e.g., metallothioneins 1 and 2), and mitochondrial DNA repair genes (e.g., OGG-1 and Neil 1) and downregulates lipogenic genes (e.g., SREBP-1) (51). A mice model of ALD demonstrated that IL-6-deficient mice were more susceptible to alcohol-induced hepatic steatosis, apoptosis, and mitochondrial DNA damage compared to wild-type animals (52). Thus, elevated IL-6 acts in a protective role in the progression of ALD by ameliorating steatosis and promoting liver regeneration (51,52). On the other hand, IL-10 is a well-documented anti-inflammatory cytokine. It was reported that IL-10-deficient mice exhibit increased liver inflammation and less steatosis after being fed alcohol or a high-fat diet (53). Those papers also proposed that IL-10 plays a dual role in regulating alcoholic fatty liver and liver injury (54). First, IL-10 inhibits LPS, TNF-α and complements activation, thereby reducing steatosis and liver damage. Second, IL-10 blocks IL-6 production, thereby reducing liver regeneration and enhancing steatosis and liver damage (54). In our study, hepatic IL-6 and IL-10 levels significantly increased in rats fed an ethanol-containing diet (E group). This result is consistent with previous animal studies of ALD. However, when dietary fatty acids of the ethanol-containing diet were high in SFAs, hepatic IL-6 and IL-10 levels significantly decreased in rats of EHS group. Thus, we surmised that high-dietary SFAs may inhibit increases in hepatoprotective and anti-inflammatory cytokines induced by ethanol feeding such as IL-6 and IL-10 in rats.

Conclusions

Chronic ethanol feeding for 8 weeks caused fatty changes and hepatic inflammation in rat livers. In addition, combining alcohol and saturated fats led to less fatty changes in rat livers; however, hepatic fibrosis was observed. We assumed that SFAs may improve hepatic fatty acid oxidation via increasing the expression of PPAR-α to inhibit hepatic fat accumulation, and increase the plasma TGF-β level to induce hepatic fibrosis. Moreover, chronic ethanol exposure together with saturated fats may contribute to decreased IL-6 and IL-10 levels which protect against ALD.

Acknowledgements

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