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Fat and carbohydrate in western diet contribute differently to hepatic lipid accumulation

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ABSTRACT

We investigated the contributions of dietary fat and dietary carbohydrate to the development of fatty liver induced by western diet (WD). Compared with WD-fed wild type (WT) mice, livers of WD-fed ChREBP$^{-/-}$ mice showed lipid droplets of varying sizes around the hepatic lobules, while hepatic triglyceride and cholesterol contents were only modestly decreased. Inflammation and fibrosis were suppressed in ChREBP$^{-/-}$ mice. In addition, compared with WD-fed WT mice, ChREBP$^{-/-}$ mice showed decreased β-oxidation, ketogenesis and FGF21 production, increased intestinal lipid absorption, and decreased VLDL secretion. These findings suggest that dietary fat and carbohydrate contribute differently to the development of fatty liver.

1. Introduction

High fat/high carbohydrate diets, i.e., a "western diet," are associated with non-alcoholic fatty liver disease (NAFLD), which is characterized by hepatic lipid accumulation not due to excess alcohol intake [1–4]. The pathophysiology of NAFLD involves (1) increased *de novo* synthesis of fatty acids in hepatocytes, (2) retention of lipids due to impaired hepatocyte apolipoprotein secretion, (3) β-oxidation of fatty acids and (4) excess dietary fat and carbohydrate intake [1–4]. Excess dietary glucose is primarily converted into triglyceride (TAG). *De novo* lipogenesis is regulated by insulin and glucose through activation of SREBP1c and carbohydrate response element binding protein (ChREBP), respectively [3,5–7]. ChREBP contributes in about half of the hepatic *de novo* lipogenesis [3,5–7]. Moreover, we and other groups have previously reported that ChREBP deletion in *ob/ob* mice improves weight gain, glucose intolerance, and fatty liver [8,9]. Considered together with the finding that fatty acid suppresses ChREBP transactivity through AMPK activation in hepatocytes [10,11], ChREBP may well act as an important mediator of dietary carbohydrate action in the development of fatty liver.

Here we examine the roles of dietary fat and dietary glucose on hepatic steatosis using ChREBP knockout mice. Some groups have compared the effects of feeding a high fat diet or a high carbohydrate diet on the development of NAFLD. However, an entirely carbohydrate diet or high fat diet is rarely taken, while a high fat/high carbohydrate diet, i.e. a western diet (WD), is often chosen. To better understand the mechanism of fatty liver development induced by WD, we investigated the roles of dietary fat and dietary carbohydrate in WD feeding with particular attention to ChREBP action.

2. Materials and methods

2.1. Animals, western diet feeding and tissue preparation

All animal care was approved by the Animal Care Committee of the University of Gifu. Mice were housed at 23 °C on a 12-h light/dark cycle. ChREBP$^{-/-}$ mice were backcrossed for at least 10 generations into the C57BL/6J background [12]. Male mice were used for all studies, and all experiments were performed using littermates. Mice had free access to water and were fed an autoclaved CE-2 diet (CLEA Japan, Tokyo, Japan) as the normal diet (ND). A high fat/high carbohydrate/high cholesterol diet called WD (western

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diet) was purchased from Research Diets (New Brunswick, NJ, USA). The proportions of calories derived from nutrients were as follows: 28.9 kcal% protein, 12.0 kcal% fat, 59.1 kcal% carbohydrate with 3.4 kcal/g energy density for ND and 17 kcal% protein, 41 kcal% fat, 42 kcal% carbohydrate with 4.7 kcal/g energy density for WD. WD also contained 34 g% sucrose and 1.0 g% cholesterol. Wild type (WT) and ChREBP$^{-/-}$ mice were separated and housed three mice per cage. Body weight was measured weekly between the ages of 7 and 21 weeks. Beginning at 8 weeks of age, the normal chow diet was discontinued and the mice were fed WD. Mice were killed at 21 weeks of age by cervical dislocation. All tissue samples were immediately placed into liquid nitrogen and stored at −80 °C until further analysis for hepatic TAG and cholesterol content and for quantitative PCR.

2.2. Measurement of liver triglyceride and cholesterol content and plasma profile

Liver lipids were extracted using the method of Bligh and Dyer [13] and measured using Triglyceride E-test (Wako Pure Chemicals, Osaka, Japan) and Cholesterol E-test (Wako). Blood plasma was collected from the retro-orbital venous plexus *ad libitum* or after a 6-h fast. Blood glucose and beta-hydroxybutyrate (β-OHB) were measured using a FreeStyle Freedom monitoring system (Nipro, Osaka, Japan). Plasma insulin, free fatty acid, fibroblast growth factor 21 (FGF21), triglyceride, and total cholesterol levels were determined using commercial assay kits as follows: mouse insulin ELISA kit (H type) (Shibayagi, Gunma, Japan), NEFA C-test (Wako Pure Chemicals, Tokyo, Japan), mouse/rat FGF21 ELISA kit (R&D Systems, Minneapolis, MN), Triglyceride E-test (Wako Pure Chemicals, Osaka, Japan), and Cholesterol E-test (Wako Pure Chemicals, Osaka, Japan).

2.3. RNA isolation and quantitative real-time PCR

Total RNA isolation, cDNA synthesis and real time PCR analysis were performed as previously described [14]. Real time PCR primers for mouse *Chrebp*, fatty acid synthase (*Fas*), liver type pyruvate kinase (*Pklr*), acyl CoA oxidase (*Acox*), *Fgf21*, peroxisome proliferator-activated receptor alpha (*Ppara*), microsomal triglyceride transfer protein (*Mttp*), sterol regulatory element-binding protein 1 c (*Srebp1c*), sterol regulatory element-binding protein 2 (*Srebp2*), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*Hmgcr*), Niemann-Pick C1 Like 1 (*Npc1l1*), Cd36, diacylglycerol acyl transferase 2 (*Dgat2*), tumor necrosis factor alpha (*Tnfa*), monocyte chemotactic protein 1 (*Mcp1*), Cd68, tissue inhibitor of metalloproteinase (*Timp*), transforming growth factor beta 1 (*Tgfb1*), collagen 1 (*Col1*) and RNA polymerase II (*Pol2*) were previously reported [14–21]. All amplifications were performed in triplicate. The relative amounts of mRNA were calculated using the comparative CT method. Expression of Pol2 was used as an internal control.

2.4. Intestinal lipid absorption test and VLDL secretion test

Intestinal lipid absorption test and VLDL secretion test were performed according to previously reported papers [22,23]. Briefly, 200 μl olive oil was orally administered to 18 h-starved mice 30 min after administration of 500 mg/kg BW tyloxapol (Sigma). As with the VLDL secretion test, 500 mg/kg BW tyloxapol was administered intra-peritoneally to 5 h-starved mice. Blood sampling was performed at the indicated times. The intestinal lipid absorption rate (mg/dl/h) was calculated as TG at 3 h − TG at 2 h, because plasma TG and time (h) have a good linear correlation ($R^2 = 0.99$) over 1–4 h. Similarly, the VLDL secretion rate (mg/dl/h) was calculated as TG at 3 h − TG at 2 h, because of a good linear correlation ($R^2 = 0.98$) over 1.5–3.0 h.

2.5. Statistical analysis

All values are presented as mean ± standard deviation. Data were analyzed using Student's *t*-tests. A *p*-value <0.05 was considered statistically significant.

3. Results

3.1. ChREBP deletion fails to alleviate WD diet-induced fatty liver

WT and ChREBP$^{-/-}$ mice were fed with ND and WD. As anticipated, WD feeding induced obesity in WT mice. In contrast, the average body weight of WD-fed ChREBP$^{-/-}$ mice was lower than that in wild type and in ND-fed ChREBP$^{-/-}$ mice (ND-fed WT mice 30.35 ± 2.27 g, ND-fed ChREBP$^{-/-}$ mice 30.68 ± 2.42 g, WD-fed WT mice 35.91 ± 3.48 g, BW, WD-fed ChREBP$^{-/-}$ mice 29.71 ± 2.69 g (Supplementary Table 1). WD-fed ChREBP$^{-/-}$ mice exhibited increased hepatomegaly (ND-fed WT mice 4.52 ± 0.36% BW, ND-fed ChREBP$^{-/-}$ mice 5.2 ± 0.5% BW, WD-fed WT mice 5.69 ± 0.45% BW, WD-fed ChREBP$^{-/-}$ mice 8.24 ± 0.31% BW) and lower adiposity compared with WD-fed WT mice (Supplementary Table 1). Compatible with these findings, the food intake of WD-fed ChREBP$^{-/-}$ mice was much lower than that of WD-fed WT mice (ND-fed WT mice 3.42 ± 0.32 g, ND-fed ChREBP$^{-/-}$ mice 3.83 ± 0.35 g, WD-fed WT mice 3.24 ± 0.80 g, WD-fed ChREBP$^{-/-}$ mice 2.85 ± 0.32 g) (Supplementary Table 1). Livers of WT mice had histologically small fat droplets near the central vein and large fat droplets near the portal vein (Fig. 1A). In contrast, livers of ChREBP$^{-/-}$ mice had lipid droplets of various sizes around all of the hepatic lobules (Fig. 1A). In addition, liver triglyceride and cholesterol content in ChREBP$^{-/-}$ mice were lower than those in WT mice (Fig. 1B and C). These data suggest the development of hepatic steatosis in WD-fed ChREBP$^{-/-}$ mice.

3.2. Decreased β−oxidation and ketogenesis in WD-fed ChREBP$^{-/-}$ mice

We then examined the effects of ChREBP on β−oxidation and ketogenesis. After 24 h starvation, plasma glucose levels of WD-fed ChREBP$^{-/-}$ mice exhibited a tendency to be lower than those of WD-fed WT mice (Fig. 2A). Moreover, plasma insulin levels of WD-fed ChREBP$^{-/-}$ mice were significantly lower than those of WD-fed WT mice (Fig. 2B). In WD-fed ChREBP$^{-/-}$ mice, the HOMA-R insulin sensitivity index was significantly improved (ND-fed WT mice 0.90 ± 0.42, ND-fed ChREBP$^{-/-}$ mice 0.77 ± 0.40, WD-fed WT mice 3.87 ± 0.87, WD-fed ChREBP$^{-/-}$ mice 0.93 ± 0.77) [24]. In addition, in WD-fed ChREBP$^{-/-}$ mice, 24 h-fasted FFA levels tended to be lower than those in WT mice (Fig. 2C) and 24 h-fasted plasma β-OHB levels were lower than those in WT mice (Fig. 2D). Furthermore, plasma FGF21 levels were lower in WD-fed ChREBP$^{-/-}$ mice than those in WD-fed WT mice (Fig. 2E). Consistent with these findings with respect to plasma β-OHB and FGF21 levels, *Acox*, *Ppara*, and *Fgf21* mRNA tended to be decreased in WD-fed ChREBP$^{-/-}$ mice (Fig. 2F).

3.3. Increased intestinal lipid absorption and decreased VLDL formation in WD-fed ChREBP$^{-/-}$ mice

Plasma TG levels in ND and WD-fed ChREBP$^{-/-}$ mice were lower than those in ND and WD-fed WT mice. In contrast, plasma cholesterol levels in WT mice were similar to those of ChREBP$^{-/-}$ mice (Fig. 3A and B).

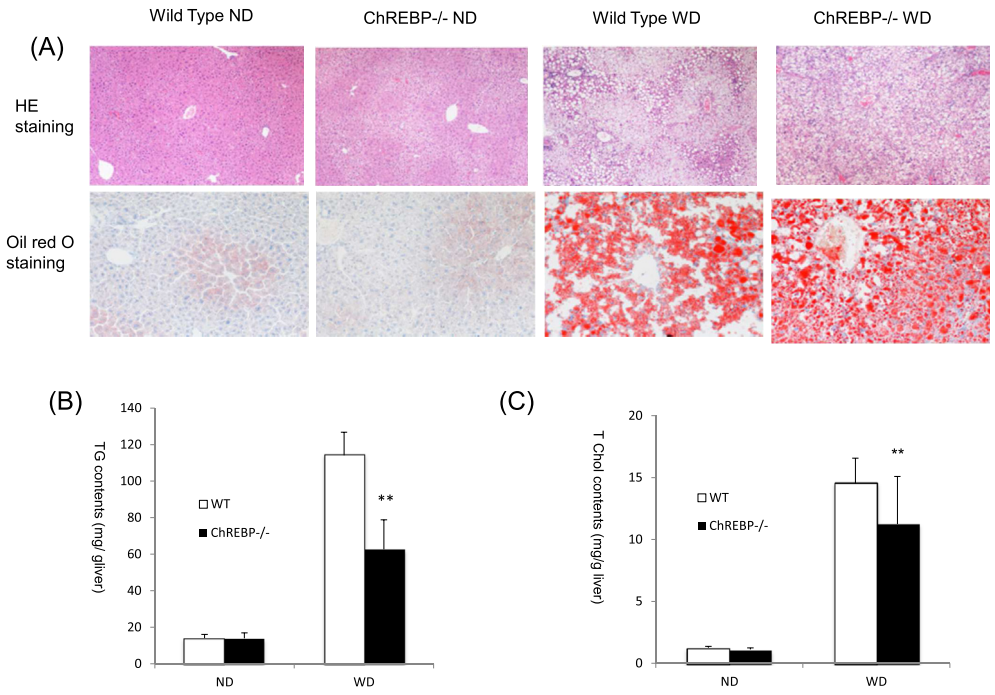

Fig. 1. ChREBP deletion fails to alleviate WD-induced fatty liver. (A) Histological analysis of liver section. Upper panel, hematoxylin eosin (HE) staining (original magnification 20x); lower panel, oil red (O) staining (original magnification 20x). (B) Hepatic triglyceride (TG) and (C) cholesterol (T. Chol); contents of wild type or ChREBP knockout (ChREBP $^{-/-}$) mice fed with normal diet (ND) or western diet (WD) (n = 8). All values are mean ± SD. **p < 0.05 between WD-fed WT (WT WD) and WD-fed −ChREBP$^{-/-}$ (ChREBP$^{-/-}$ WD) mice. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In addition to decreased β-oxidation, decreased VLDL secretion and increased intestinal lipid absorption also cause hepatic steatosis. We therefore performed an intestinal lipid absorption test. Interestingly, intestinal absorption rates in WD-fed ChREBP$^{-/-}$ mice were much higher than those in WD-fed WT mice (Fig. 3C). In contrast, hepatic VLDL secretion rates in WD-fed ChREBP$^{-/-}$ mice were much lower than those in WD-fed WT mice (Fig. 3D).

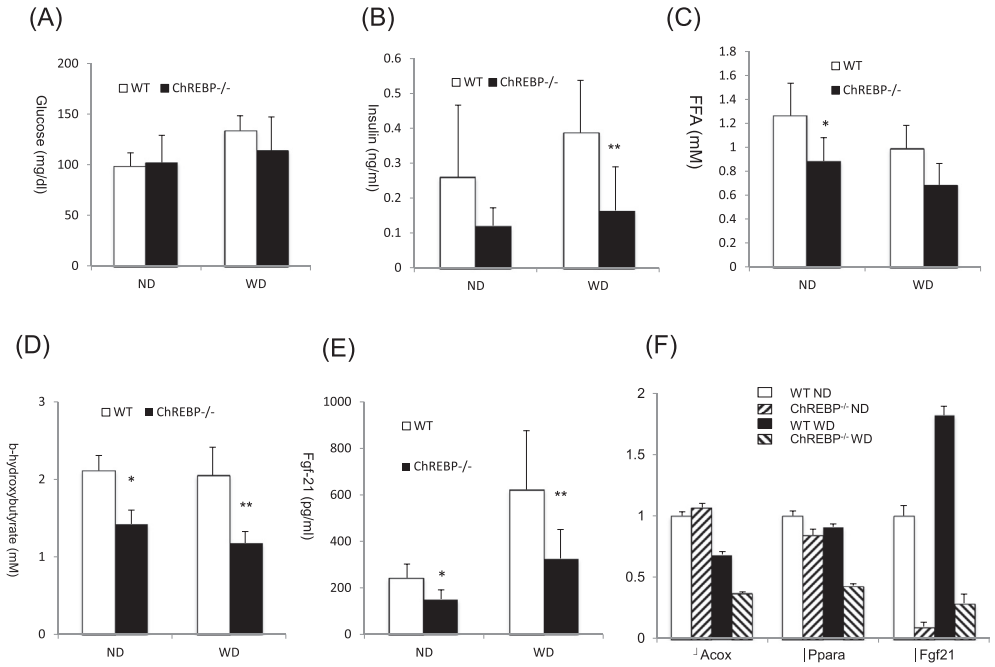

Fig. 2. Decreased ketogenesis and β−oxidation in WD-fed ChREBP$^{-/-}$ mice. Analysis of plasma levels of (A) glucose, (B) insulin, (C) free fatty acid (FFA), (D) β-hydroxybutyrate, and (E) fibroblast growth factor 21 (FGF21) of wild type or ChREBP knockout (ChREBP$^{-/-}$) mice fed with normal diet (ND) or western diet (WD) (n = 8). (F) mRNA expression analysis of acyl coA oxidase (*Acox*), peroxisomal proliferator activator α (*Ppara*), and *Fgf-21 in* livers from ND−or WD-fed WT and ChREBP$^{-/-}$ mice (n = 3) measured by real time PCR analysis. The RNA polymerase II gene was used as a control. All values are mean ± SD. *p < 0.05 between ND-fed WT (WT ND) and ND-fed −ChREBP$^{-/-}$ (ChREBP$^{-/-}$ ND) mice. **P < 0.05 between WD-fed WT (WT WD) and WD-fed −ChREBP$^{-/-}$ (ChREBP$^{-/-}$ WD) mice.

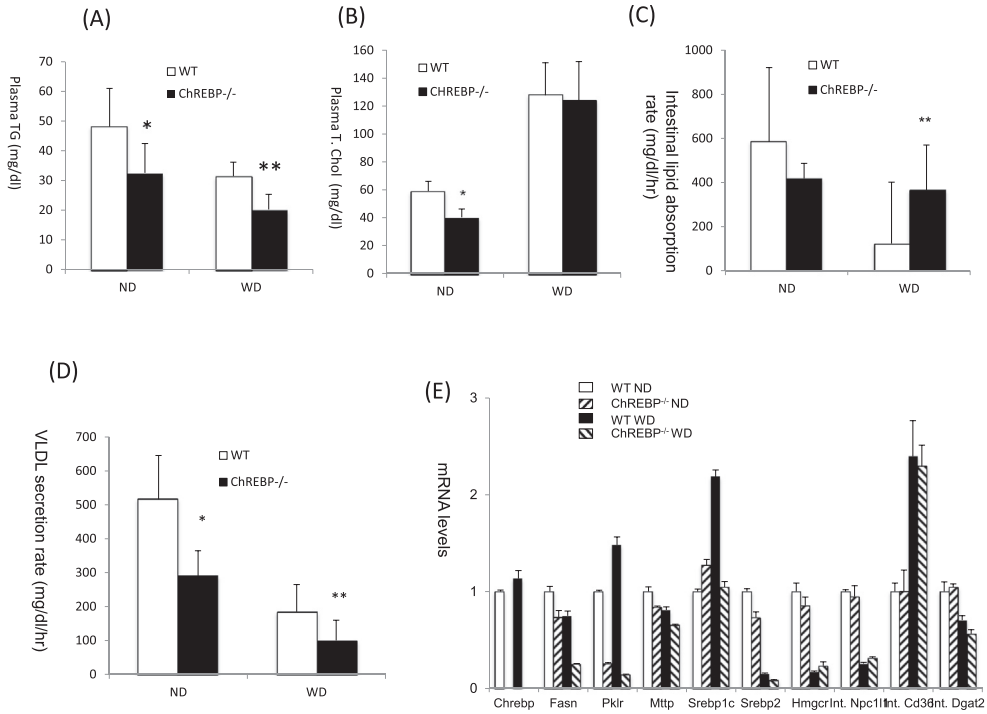

Fig. 3. Increased intestinal lipid absorption and lower hepatic VLDL secretion in WD-fed ChREBP$^{-/-}$ mice, (A and B) plasma triglyceride (TG) and total cholesterol (T. Chol) levels of normal diet (ND) and western diet (WD) (n = 8). Intestinal lipid absorption rates (C) and VLDL secretion rates (D) were calculated as TG at 3hr-TG at 2hr (n = 8). The RNA polymerase II gene was used as a control. All values are mean ± SD. *p < 0.05 between ND-fed WT (WT ND) and ND-fed −ChREBP$^{-/-}$ (ChREBP$^{-/-}$ ND) mice. **p < 0.05 between WD-fed WT (WT WD) and WD-fed ChREBP$^{-/-}$ (ChREBP$^{-/-}$ WD) mice.

We then measured the mRNA expression levels of genes involved in lipid metabolism. As anticipated, *Chrebp* mRNA was undetectable in ND- and WD-fed ChREBP$^{-/-}$ mice (Fig. 3E). Consistently, mRNA levels of the ChREBP target genes *Pklr*, *Fasn*, and *Mttp* tended to be remarkably decreased in WD-fed ChREBP$^{-/-}$ mice (Fig. 3E). In accord with the decreased plasma insulin levels in WD-fed ChREBP$^{-/-}$ mice, Srebp1c mRNA levels of WD-fed ChREBP$^{-/-}$ mice tended to be also decreased (Fig. 3E). With respect to cholesterol, hepatic mRNA expression levels of *Srebp2* and *Hmgcr* and intestinal *Npc1l1* mRNA levels in WD-fed ChREBP$^{-/-}$ mice were similar to those in WD-fed WT mice (Fig. 3E). Cd36 and *Dgat2* mRNA levels of WD-fed ChREBP$^{-/-}$ mice also were similar to those of the WD-fed WT mice (Fig. 3E).

3.4. ChREBP deletion protects against inflammation and fibrosis

We also examined the effects of ChREBP deletion on inflammation and fibrosis. Although WD-fed WT and ChREBP$^{-/-}$ mice had higher plasma AST and ALT levels compared with those of ND-fed WT and ChREBP$^{-/-}$ mice, no significant difference was observed between WT and ChREBP$^{-/-}$ mice (Fig. 4A and B). Moreover, while hepatic mRNA expression levels of genes involved in inflammation (*Tnfa*, *Mcp1*, and Cd68) and fibrosis (*Col1a*, *Tgfb1*, and *Timp*) were similar in ND-fed ChREBP$^{-/-}$ mice and WT mice (Fig. 4C), they tended to be much lower in WD-fed ChREBP$^{-/-}$ mice than in WD-fed WT mice (Fig. 4C).

4. Discussion

In this study, we investigated promotion by dietary fat of development of fatty liver in ChREBP$^{-/-}$ mice. Although ChREBP gene deletion somewhat improved body weight gain, hepatomegaly and fatty liver development were still observed in these mice. As compared with WD-fed WT mice, WD-fed ChREBP$^{-/-}$ mice showed (1) decreased β−oxidation, ketogenesis, and FGF21 production, (2) increased intestinal lipid absorption, (3) decreased VLDL formation, and (4) decreased mRNA expression of inflammatory and fibrotic factors. These findings suggest that suppression of ChREBP transactivation by itself is insufficient to prevent development of NAFLD. Furthermore, dietary fat and dietary carbohydrate intake make distinct contributions to the development of fatty liver induced by WD.

In this study, the hepatic distributions of TG deposits in WD-fed ChREBP−/− mice were found to be histologically distinct from those in WD-fed WT mice. One group reported that differences in fat and carbohydrate content account for the distinct histological findings regarding lipid distribution [25]. Diets high in fat tend to induce pericentral steatosis; diets high in carbohydrate or high in both carbohydrate and fat tend to induce periportal steatosis. Moreover, hepatic zonation results in heterogeneity of metabolic features, such that FFA uptake and oxidation are predominant in periportal hepatocytes while glycolysis and *de novo* lipogenesis predominate in pericentral hepatocytes. Considering that ChREBP gene deletion causes both β-oxidation and *de novo* lipogenesis to decrease [10], it might well contribute to the distinct distribution of TG deposits in liver. Further investigation is required to clarify the relationships between the zonation of ChREBP distribution and the metabolic consequences.

In this study, fasted plasma β-OHB levels were much lower in WD-fed ChREBP$^{-/-}$ mice, reflecting decreased β-oxidation and ketogenesis in liver of WD-fed ChREBP$^{-/-}$ mice. Moreover, plasma FGF21 levels also were remarkably decreased in both ND- and WD-fed ChREBP$^{-/-}$ mice. FGF21 is a regulator of fatty acyl CoA oxidation and ketogenesis, and is regulated by PPARα and ChREBP [16,17,26–31]. Plasma β-OHB and FGF21 levels are higher in ketogenic diet (high fat/low carbohydrate diet) (KD) fed mice [26–30] and FGF21 administration reverses hepatic steatosis and improves insulin sensitivity in diet-induced obese mice [26–30]. This

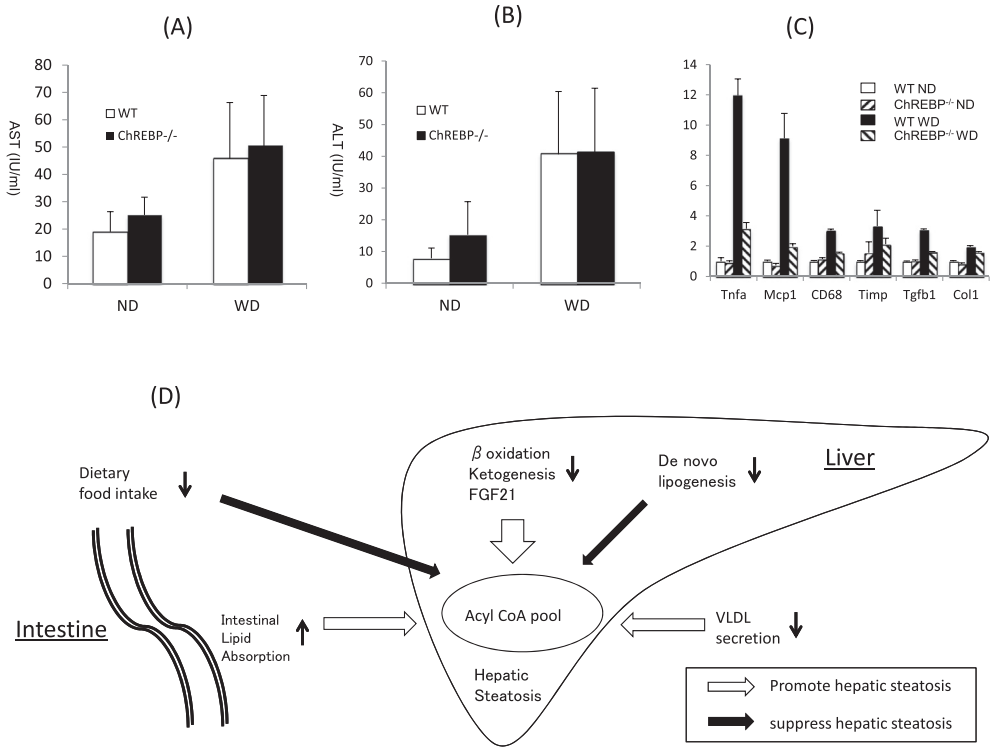

Fig. 4. ChREBP deletion protects against inflammation and fibrosis. (A and B) Plasma levels of AST and ALT of normal diet (ND) and western diet (WD) (n = 8). (C) mRNA levels of inflammation and fibrosis in ND- and WD-fed WT and ChREBP$^{-/-}$ mice (n = 3). The RNA polymerase II gene was used as control. All values are mean ± SD. *p < 0.05 between ND-fed WT (WT ND) and ND-fed ChREBP$^{-/-}$ (ChREBP−/− ND) mice. (D) Schematic presentation of the mechanism by which western diet feeding causes development of fatty liver in ChREBP$^{-/-}$ mice. In WD fed ChREBP−/− mice, decreasedβ-oxidation, ketogenesis, and FGF-21production, decreased VLDL secretion and increased intestinal lipid absorption promote hepatic lipid accumulation. In contrast, decreased food intake and decreased *de novo* lipogenesis suppress hepatic lipid accumulation.

accords with our data, in which KD-fed FGF21$^{-/-}$ mice developed hepatic steatosis and showed decreased ketogenesis [30]. Thus, decreased plasma FGF21 levels in WD-fed ChREBP$^{-/-}$ mice might readily promote hepatic steatosis and decreased ketogenesis. Considering that ChREBP directly regulates *Fgf21* gene expression [16,17,31], suppression of ChREBP activity could promote hepatic steatosis through decreased plasma FGF21 levels in addition to decreased β-oxidation and ketogenesis.

In the intestine, TAG synthesis plays a prominent role in the absorption of dietary fat [32–35]. It has been reported that high fat diet causes decreased lipid absorption [33]. Consistently, we found that WD feeding decreased the rate of lipid absorption in WT mice. In contrast, the intestinal lipid absorption rates in WD-fed ChREBP$^{-/-}$ mice were increased compared with those in WD-fed WT mice. At present, it is unclear why the intestinal lipid absorption rates in WD-fed ChREBP$^{-/-}$ mice should be higher than those in WD-fed WT mice; nevertheless, increased intestinal lipid absorption may weakly promote hepatic steatosis in WD-fed ChREBP$^{-/-}$ mice.

Mttp catalyzes the transfer of neutral lipids (TG, Pl, CE) to nascent apoB, a rate-limiting step in hepatic VLDL production [36,37]. In accord with the decreased Mttp mRNA expression, VLDL secretion also was decreased. Considering that *Mttp* is a ChREBP target gene [17], decreased *Mttp* mRNA levels are likely due to ChREBP gene deletion. Thus, decreased VLDL formation expression may also weakly promote hepatic steatosis in WD-fed ChREBP$^{-/-}$ mice.

As we and other groups have previously reported [8,9], ChREBP deletion or silencing in ob/ob mice restores the hepatic TG content in these mice to normal levels. However, in the previous studies, ob/ob ChREBP$^{-/-}$ mice were fed a normal chow diet. Compared with normal chow diet, the WD used in this study contained a larger amount of dietary fat and cholesterol. Nevertheless, in accord with these findings, in the present study, KD feeding caused fatty liver without increased hepatic *de novo* lipogenesis, whereas WD feeding caused development of fatty liver with increased *de novo* lipogenesis [38]. Considered with the histological changes observed in WD-fed ChREBP$^{-/-}$ mice, suppression of only *de novo* lipogenesis therefore may be insufficient to prevent the development of fatty liver due to excessive dietary fat intake.

Cholesterol also plays an important role in NAFLD and non-alcoholic steatohepatitis progression [39]. Importantly, dietary cholesterol intake may promote inflammation and fibrosis in liver independently of hepatic steatosis [39]. In this study, hepatic cholesterol content was lower in WD-fed ChREBP$^{-/-}$ mice than in WD-fed WT mice partly due to decreased food intake. Further investigation might be needed to perform pair-feeding diet, although difficult because of lower food intake [8,12]. AST and ALT levels in WD-fed WT and ChREBP$^{-/-}$ were similarly elevated compared with those in ND-fed WT and ChREBP$^{-/-}$ mice. However, the mRNA expression of genes involved in inflammation (*Tnfa*, *Mcp1*, and Cd68) and fibrosis (*Timp*, *Mmp2*, *Tgfb1*, and *Col1*) were much lower in WD-fed ChREBP$^{-/-}$ mice than in WD-fed WT mice. Considering that the mRNA levels of these factors are comparable in ND-fed ChREBP$^{-/-}$ and WT mice, suppression of inflammation and fibrosis may well be a secondary effect of decreased cholesterol and TG content rather than a direct effect of ChREBP gene deletion.

Although low-carbohydrate diets are used to restrict carbohydrate consumption in the treatment of obesity or diabetes [4], the effects of such diets remain extremely controversial [4]. For example, low carbohydrate, high fat diets enhance the risk of mortality and type 2 diabetes, especially when animal proteins and fats are consumed [4]. Moreover, a high fat, low carbohydrate, ketogenic diet has been shown to prevent weight gain in mice, but

causes symptoms of NAFLD [4,38]. These results are consistent with our observations in WD-fed ChREBP$^{-/-}$ mice. Further investigation of the role of dietary fat intake in the development of NAFLD is required to determine the best course of treatment of NAFLD and non-alcoholic steatohepatitis.

In conclusion, ChREBP deletion improves body weight gain, but does not completely improve hepatic steatosis. As the western diet is high in both fat and carbohydrate, suppression of ChREBP transactivation *per se* may be insufficient to prevent the development of fatty liver. Considering that dietary fat and dietary carbohydrate promote the development of fatty liver in different manners, restriction of both dietary fat and carbohydrate intake are required for the prevention of fatty liver.

Conflict of interest

The authors state that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.04.092.

Transparency document

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