Molecules and Cells

Increased Hepatic Lipogenesis Elevates Liver Cholesterol Content

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Cardiovascular diseases (CVDs) are the most common cause of death in patients with nonalcoholic fatty liver disease (NAFLD) and dyslipidemia is considered at least partially responsible for the increased CVD risk in NAFLD patients. The aim of the present study is to understand how hepatic *de novo* lipogenesis influences hepatic cholesterol content as well as its effects on the plasma lipid levels. Hepatic lipogenesis was induced in mice by feeding a fat-free/high-sucrose (FF/HS) diet and the metabolic pathways associated with cholesterol were then analyzed. Both liver triglyceride and cholesterol contents were significantly increased in mice fed an FF/HS diet. Activation of fatty acid synthesis driven by the activation of sterol regulatory element binding protein (SREBP)-1c resulted in the increased liver triglycerides. The augmented cholesterol content in the liver could not be explained by an increased cholesterol synthesis, which was decreased by the FF/HS diet. HMG-CoA reductase protein level was decreased in mice fed an FF/HS diet. We found that the liver retained more cholesterol through a reduced excretion of bile acids, a reduced fecal cholesterol excretion, and an increased cholesterol uptake from plasma lipoproteins. Very low-density lipoprotein-triglyceride and -cholesterol secretion were increased in mice fed an FF/HS diet, which led to hypertriglyceridemia and hypercholesterolemia in *Ldlr*$^{-/-}$ mice, a model that exhibits a more human like lipoprotein profile. These findings suggest that dietary cholesterol intake and cholesterol synthesis rates cannot only explain the hypercholesterolemia associated with NAFLD, and that the control of fatty acid synthesis should be considered for the management of dyslipidemia.

Keywords: cholesterol, dyslipidemia, fatty liver, lipogenesis

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most common liver condition with a prevalence of ~33% among the adult population in the US (Browning et al., 2004). NAFLD is associated with an increased incidence of cardiovascular diseases (CVDs) (Anstee et al., 2013; Targher et al., 2010) and patients with NAFLD are more likely to die from CVD than liver disease (Chatrath et al., 2012; Cohen and Fisher, 2013). Atherogenic dyslipidemia, which is characterized by an increased level of plasma triglycerides, decreased level of high-density lipoprotein (HDL), and the presence of small and dense low-density lipoprotein (LDL) particles, is considered at least partially responsible for the increased CVD risk in NAFLD patients (Cohen and Fisher, 2013). NAFLD is strongly associated with obesity and insulin resistance, which are also known risk factors for dyslipidemia (Angulo, 2002). However, hepatic steatosis was independently associated with atherogenic dyslipidemia after adjustment for obesity, physical activity, hyperglycemia, and systemic inflammation (Makadia et al., 2013). The pathogenesis of dyslipidemia in NAFLD is not completely understood, but hepatic overproduction of very low-density

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lipoprotein (VLDL), alteration in lipoprotein lipase activity, and dysregulated clearance of lipoproteins are considered major contributors (Chatrath et al., 2012; Howard, 1987; Semenkovich, 2006). Understanding the pathophysiology of the dyslipidemia associated with NAFLD would guide better strategies to reduce morbidity and mortality in patients with NAFLD.

In the liver, triglycerides come from the diet, *de novo* fatty acid synthesis, and free fatty acids released from the adipose tissue (Cohen et al., 2011). Sterol regulatory element binding protein (SREBP)-1c is the principle transcription factor that activates genes required for *de novo* fatty acid synthesis and the first step of triglyceride synthesis (Horton et al., 2002). In hepatocytes, cholesterol exists as either free cholesterol or cholesteryl ester. Cholesterol is an essential component of cellular membranes and a precursor for bile acids and steroid hormones (Ikonen, 2008). Although most cells synthesize cholesterol, the liver plays a central role in cholesterol synthesis and homeostasis. Cholesterol is synthesized from acetyl-CoA through a cascade of reactions performed by enzymes that are regulated by SREBP-2. SREBP-2 also activates the genes encoding the LDL receptor (LDLR) and protein convertase subtilisin/kexin type 9 (PCSK9), a secreted protein that degrades hepatic LDLRs, thereby reducing cholesterol uptake (Horton et al., 2002; 2003; 2009).

The role of SREBP-1c activation in the development of hypertriglyceridemia has been studied in different animal models (Horton et al., 1999; Moon et al., 2012; Okazaki et al., 2010). In hamsters, activation of SREBP-1 by a high sucrose diet induced hypertriglyceridemia (Moon et al., 2012). They also exhibited hypercholesterolemia, characterized by a substantial increase in VLDL-cholesterol levels. In a mouse model with a *Ldlr*$^{-/-}$ background that overexpressed the nuclear form of SREBP-1a in the liver (Tg-SREBP1a; *Ldlr*$^{-/-}$), the expression of both fatty acid and cholesterol synthesis pathway-related genes was highly elevated because SREBP-1a can induce genes associated with both pathways (Horton and Shimomura, 1999). The hypercholesterolemia observed in the Tg-SREBP1a; *Ldlr*$^{-/-}$ mice could be at least partly due to the elevated cholesterol synthesis in the liver (Horton et al., 1999). *Ldlr*$^{-/-}$ mice treated with an LXR activator (T0901317), which induces SREBP-1c expression, also developed dyslipidemia, similar to that of Tg-SREBP1a; *Ldlr*$^{-/-}$ mice (Okazaki et al., 2010). Massive hypercholesterolemia and hypertriglyceridemia with chylomicron-sized VLDL developed, which were nearly abolished when *Srebp-1c* was deleted. While SREBP-1c activation was the primary reason for hypertriglyceridemia, the exact mechanisms for hypercholesterolemia were not fully investigated.

In the present study, we determined how hepatic *de novo* lipogenesis influences hepatic cholesterol content and metabolic pathways, as well as its effects on plasma lipid levels. Hepatic lipogenesis was induced in mice by feeding an FF/HS diet. Hepatic cholesterol synthesis and the associated metabolic pathways were then analyzed. We show that liver cholesterol content was elevated in the liver not by increased cholesterol synthesis, but by a decrease in bile acids and fecal cholesterol excretion, and an increase in cholesterol uptake from lipoproteins. Increased hepatic fat resulted in hypercholesterolemia with hypertriglyceridemia in *Ldlr*$^{-/-}$ mice.

MATERIALS AND METHODS

Animals and diet

Males C57BL/6J wild-type and *Ldlr*$^{-/-}$ mice were purchased from The Jackson Laboratories (USA). Mice were housed in colony cages with free access to food and water under a 12-h light/12-h dark cycle in a temperature-controlled environment. Mice were fed a chow diet obtained from Harlan Teklad Premier Laboratory Diets (USA) (Teklad Mouse/Rat Diet 2018) or a fat-free/high-sucrose (FF/HS) diet containing 60% sucrose (w/w) (Fat Free, Modified, Cat No. 960238; MP Biomedicals, USA) for 20 days. Mice were fed only during the dark cycle (7:00 P.M. to 7:00 A.M.) for the last 3 days prior to the experiments. All experiments were conducted using 10- to 12-week-old male mice. All animal studies were approved by the Institutional Animal Care & Use Committee at the University of Texas Southwestern Medical Center (APN#2015-101135).

Metabolic parameters

Blood was obtained from the tail vein of restrained animals in heparin-coated tubes and plasma was separated and stored at −80°C. Plasma cholesterol and triglyceride concentrations were determined using Infinity Cholesterol Liquid Stable Reagent and Infinity Triglycerides Liquid Stable Reagent (Thermo Fisher Scientific, USA), respectively. Plasma lipoprotein profiles were obtained for each group by pooling equal amounts of plasma from each mouse. After filtration (Costar; Corning, USA), 20 μl of each plasma sample was pooled to a total of 200 μl per group, and the pooled plasma sample was injected into the fast protein liquid chromatography (FPLC) system. FPLC analysis was performed at 4°C using a Superose 6 10/300 GL exclusion column at a flow rate of 0.5 ml/min. Cholesterol and triglyceride concentrations in the collected fractions were then determined. Plasma Apo-CIII concentration was measured by the APOC3 (mouse) ELISA Kit (Abnova, USA). Bile was collected from the gallbladder after 3 h of fasting, and bile acid concentrations were measured using the Colorimetric Total Bile Acid Assay Kit from Diazyme Laboratories (USA).

Radiolabeling of human lipoproteins

Human LDL (1.019-1.063 g/ml) and HDL (1.125-1.215 g/ml) were prepared as described previously (Goldstein et al., 1983). The purity of each lipoprotein was validated by FPLC. Human LDL or HDL was labeled with cholesteryl oleate [cholesteryl-1,2-^{3}H(N)] (Perkin Elmer, USA) as described previously (May et al., 2013).

Lipid uptake and plasma clearance

Mice were anesthetized using pentobarbital (80 mg/kg of body weight) and ^{3}H-cholesteryl oleate-labeled LDL or HDL (10^{6} cpm/mouse) was injected via the penile vein. Blood was collected before injection (0 min) and at 2, 15, 30, and 60 min after injection. Plasma was separated, and triglyceride and cholesterol concentrations were measured as described above. Radioactivity in 20 μl of the plasma was measured.

One hour after injection, mice were euthanized with isoflurane and perfused with saline. The liver was collected, washed with saline, and the whole liver was dissolved in 3 ml of 50% KOH in ethanol. The ^{3}H-labelled cholesterol content of the liver was measured.

In vivo VLDL secretion

Tyloxapol was purchased from Sigma-Aldrich (USA); 10% tyloxapol solution was prepared using saline. Mice were fasted for 6 h and injected with tyloxapol (500 mg/kg) via the penile vein. Blood was collected from the tail vein before injection (0 min) and at 15, 30, 60, 90, 120, and 180 min after injection. Plasma was separated, and triglyceride and cholesterol concentrations were determined. Triglyceride and cholesterol secretion rates were calculated from the linear regression analysis of time versus concentration.

Fatty acid and cholesterol synthesis *in vivo*

The synthesis rates of sterol and fatty acids in the liver were determined in mice using ^{3}H-labeled water as described previously (Shimano et al., 1996).

Preparation of primary hepatocytes and measurement of lipid synthesis rates

Hepatocytes were isolated from C57BL/6J wild-type mice and cultured as described previously (Kim et al., 2020; Matsuda et al., 2001). After a 3-h attachment period, the cells were incubated with 0.5 mM ^{14}C-sodium acetate for 4 h. Lipids were extracted, and then, fatty acids, cholesterol, cholesteryl ester, and triglycerides were separated by TLC as described previously (Matsuda et al., 2001; Moon et al., 2009). Incorporation of ^{14}C-acetate into newly synthesized lipids was then determined.

Fecal cholesterol excretion

The amount of cholesterol in feces was measured as described previously, with slight modifications (Turley et al., 1996). Mice were housed in individual cages a week before the experiment, and feces were collected every 24 h for three days. Feces were dried completely, ground, and resuspended in water (100 μg/2 ml). Lipids were extracted in chloroform/methanol (2:1) and resuspended in isopropanol. Cholesterol concentrations in the extracts were measured using Infinity Cholesterol Liquid Stable Reagent (Thermo Fisher Scientific).

Live cell imaging

Hepatocytes isolated from C57BL/6J wild-type mice were seeded at a density of 1×10^{6} cells in a 60-mm dish. After a 2-h attachment period, the cells were washed and incubated with 1 μg/ml of BODIPY 493/503 (Thermo Fisher Scientific) and 15 μg/ml of dehydroergosterol (DHE; Sigma-Aldrich) in either the presence or absence of 20 μM oleic acid for 24 h. After incubation, the cells were imaged with a ZEISS LSM 780 confocal microscope (Zeiss, USA).

Immunoblot analysis

Membrane and nuclear proteins were prepared individually from frozen livers, and equal amounts of protein from each mouse of the same group were pooled as previously described (Engelking et al., 2004). Aliquots of pooled proteins were subjected to SDS-PAGE on 8% gels and transferred to a nitrocellulose membrane (Bio-Rad, USA). Immunoblot analyses were performed using polyclonal anti-mouse SREBP-1, SREBP-2, and HMG-CoA reductase antibodies as previously described (Rong et al., 2017). Anti-Scavenger receptor class B type 1 (SR-BI) antibody was obtained from Abcam (USA) and anti-cluster of differentiation 36 (CD36) antibody was obtained from R&D Systems (USA). Anti-mouse cAMP response element binding protein (CREB; Invitrogen, USA) and anti-dog calnexin (Enzo Life Science, USA) antibodies were used as loading controls for nuclear and membrane proteins, respectively. Signals were detected using the SuperSignal West Pico Chemiluminescent Substrate System (Thermo Fisher Scientific) and visualized with Odyssey CLx Imaging system and LI-COR Image Studio™ software (LI-COR, USA).

Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was prepared from mouse livers with an RNA STAT-60 kit (Tel-Test, USA), and qPCR was performed as previously described (Rong et al., 2017). All reactions were performed in triplicate, and the relative amount of all mRNAs was calculated using the comparative threshold cycle (C_T) method. Mouse ApoE was used as the invariant control. The Sequences of the primers for qPCR are shown in Supplementary Table S1.

Statistics

All results are reported as the mean ± SE. Statistical significance was analyzed using a Student's *t*-test (Prism 9; GraphPad Software, USA). *P* values < 0.05 were considered statistically significant.

RESULTS

FF/HS diet increases liver cholesterol content despite reduced cholesterol synthesis

FF/HS diets have been previously used to induce hepatic *de novo* lipogenesis (Iritani et al., 1992; Kim and Freake, 1996; Moon et al., 2012; Weigand et al., 1980). Here, the effects of *de novo* lipogenesis on overall lipid metabolism in C57BL/6J wild-type mice fed an FF/HS diet for 20 days were compared with those in mice fed a chow diet. Plasma glucose, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels were not different between the groups (Fig. 1A). As previously reported (Moon et al., 2012), liver triglyceride content significantly increased in the mice fed an FF/HS diet (Fig. 1B). SREBP-1c is a major transcription factor that regulates the expression of lipogenic genes. The expression of both the precursor and the nuclear form of SREBP-1 was elevated in the livers of the mice fed an FF/HS diet (Fig. 1C). Consistent with the SREBP-1 protein levels, the mRNA expression of SREBP-1c and its known target genes, such as ATP-citrate lyase (*Acl*), acetyl-CoA carboxylase-α (*Acaca*), fatty acid synthase (*Fasn*), elongation of long chain fatty acids 6 (*Elovl6*), and stearoyl-CoA desaturase-1 (*Scd-1*), showed a 1.6- to 4.6-fold elevation (Supplementary Fig. S1A). Fatty acid synthesis rates were measured *in vivo* in these mice. ^{3}H-labelled water was injected into the mice, their hepatic lipids were extract-

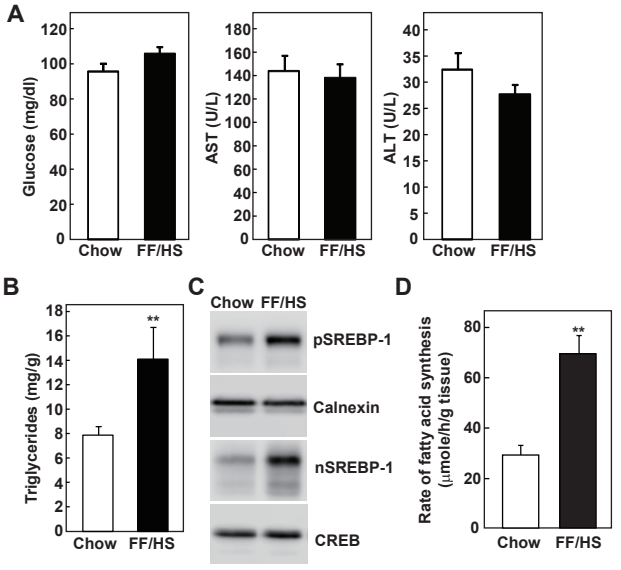

Fig. 1. Hepatic triglyceride content and synthesis rates in mice fed a chow or an FF/HS diet. (A) Plasma glucose, AST, ALT levels were measured in mice fed a chow or an FF/HS diet for 20 days. (B) Hepatic triglyceride contents were measured. Data represents the mean ± SE of 10 mice. (C) Membrane and nuclear proteins were prepared individually from livers. Equal amounts of proteins from 10 mice of the same group were pooled and immunoblot analysis of SREBP-1 was performed. Calnexin and CREB were used as loading controls for membrane and nuclear proteins, respectively. (D) *In vivo* synthesis rates of fatty acids were measured in livers of five mice fed with chow or an FF/HS diet. Mice were injected intraperitoneally with ^{3}H-labeled water. One hour after the injection, incorporation of ^{3}H-labeled water into the newly synthesized fatty acids was measured in the liver. The data represents the mean ± SE of 5 mice. $**P < 0.01$ by Student's *t*-test.

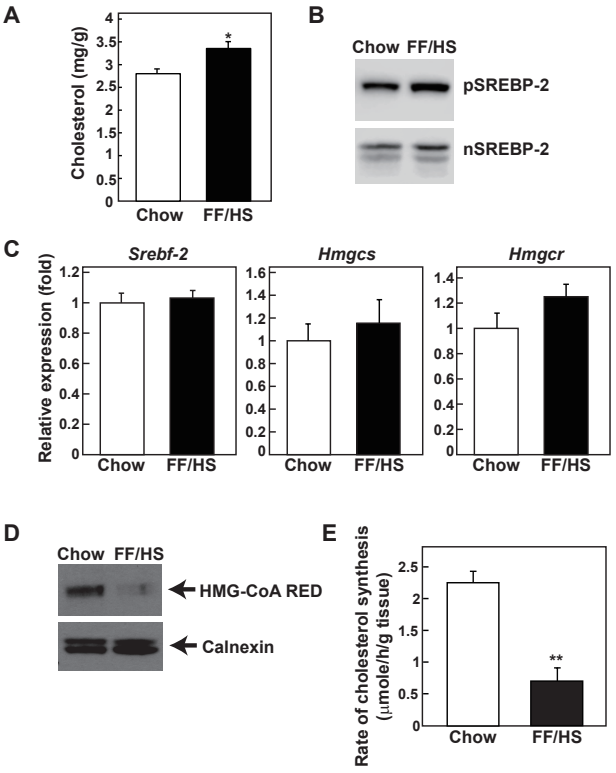

Fig. 2. Hepatic cholesterol content and synthesis rates in mice fed a chow or an FF/HS diet. (A) Cholesterol contents were measured in the liver of mice fed a chow or an FF/HS diet for 20 days. Data represents the mean ± SE of 10 mice. (B) Membrane and nuclear proteins presented in Fig. 1 were used for immunoblot analysis of SREBP-2. (C) mRNA expression of genes involved in cholesterol synthesis were determined. Values represent the mean ± SE of 10 mice relative to mice fed a chow diet, which is defined as 1. *Apoe* was used as the invariant control. (D) Equal amounts of membrane proteins prepared individually from 6 mice of the same group were pooled and immunoblot analysis of HMG-CoA reductase was performed. Calnexin was used as a loading control. (E) *In vivo* synthesis rates of cholesterol were measured in livers of mice fed with a chow or an FF/HS diet. Mice were injected intraperitoneally with ^{3}H-labeled water. One hour after the injection, the incorporation of ^{3}H-labeled water into newly synthesized cholesterol was measured in the liver. Each bar represents the mean ± SE of the values from 5 mice. $*P < 0.05$, $**P < 0.01$ by Student's *t*-test.

ed, and the incorporation of ^{3}H-water into the newly synthesized fatty acids was measured. Consistent with the level of the nuclear form of SREBP-1 and the expression levels of its target genes, the rate of fatty acid synthesis was elevated in the liver of the mice fed an FF/HS diet (Fig. 1D). These results confirmed that the FF/HS diet activated SREBP-1c, thereby inducing the expression of genes involved in fatty acid synthesis, which resulted in an increase in fatty acid synthesis and an accumulation of triglycerides in the liver.

Cholesterol content was also significantly increased in the livers of mice fed an FF/HS diet (Fig. 2A). All genes encoding enzymes responsible for cholesterol synthesis are regulated by SREBP-2 (Horton et al., 2002); therefore, SREBP-2 and its target genes were analyzed. While the precursor form of SREBP-2 appeared slightly increased, the amount of the transcriptionally active nuclear form was not altered by the FF/HS diet (Fig. 2B). The mRNA expression levels of neither SREBP-2 (*Srebf-2*) nor the SREBP-2 target genes such as HMG-CoA synthase (*Hmgcs*) and HMG-CoA reductase (*Hmgcr*) were changed by the FF/HS diet (Fig. 2C). HMG-CoA reductase is the rate limiting enzyme in the cholesterol synthesis pathway and is regulated at the transcriptional, post-transcriptional, and post-translational levels (DeBose-Boyd, 2008). Despite no change in the mRNA level, we observed that the expression of the HMG-CoA reductase protein was significantly reduced in the liver of the mice fed an FF/HS diet (Fig. 2D), indicating a post-transcriptional regulation of the enzyme, such as ER-associated degradation (Johnson and DeBose-Boyd, 2018). Next, *in vivo* cholesterol synthesis rates were measured by determining the incorporation of ^{3}H-water to the newly synthesized hepatic sterols. Consistent with the HMG-CoA reductase protein level, sterol synthesis rates were significantly reduced in the livers of the mice fed an FF/HS diet (Fig. 2E). These results suggest that following the administra-

tion of the FF/HS diet, cholesterol contents were increased. This could not be explained because of an increased cholesterol synthesis which was decreased due to the reduction of HMG-CoA reductase protein expression. As a result of the increased hepatic cholesterol content induced by the FF/HS diet, the post-transcriptional regulation of HMG-CoA reductase might be involved.

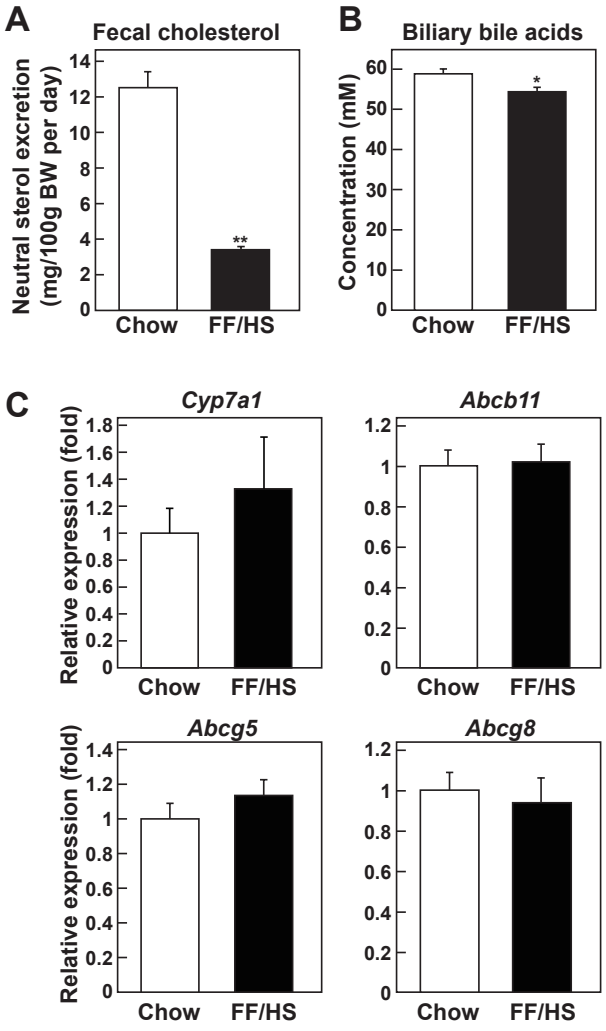

Fig. 3. Fecal cholesterol content and biliary bile acid concentration in mice fed a chow or an FF/HS diet. (A) Mice were fed a chow or an FF/HS diet for 20 days. For the last 3 days of the experiment, feces were collected from each mouse, and cholesterol concentrations were measured. Values represent the mean ± SE of 10 mice. (B) Bile acid concentrations were measured in each mouse following a 4-h fast. Values represent the mean ± SE of 10 mice. (C) mRNA expression of genes involved in bile acid synthesis (*Cyp7a1*), bile acid excretion (*Abcb11*), and cholesterol excretion (*Abcg5* and *Abcg8*) were measured in the livers of mice fed a chow or an FF/HS diet. Values represent mean ± SE of 10 mice, relative to those of mice fed a chow diet, which are defined as 1. *Apoe* was used as the invariant control. $*P < 0.05$, $**P < 0.01$ by Student's *t*-test.

Cholesterol excretion was reduced in the mice fed an FF/HS diet

To determine why the liver cholesterol content was increased despite the reduced cholesterol synthesis in the mice fed an FF/HS diet, additional metabolic pathways involved in cholesterol homeostasis were examined. In the liver, cholesterol can be excreted into the bile directly as free cholesterol or after its conversion into bile acids. Feces contains cholesterol from bile, diet, and transintestinal cholesterol excretion. Both diets used in this study are cholesterol-free; therefore, the cholesterol found in the feces must be endogenous. Feces of mice fed a chow or an FF/HS diet were collected for 3 days at the end of the diet feeding period, and fecal cholesterol contents were measured. Fecal cholesterol content of mice fed an FF/HS diet was reduced by 73% compared to mice fed a chow diet (Fig. 3A). Bile acid concentrations were also significantly reduced in the bile of mice fed the FF/HS diet, compared to the mice fed the chow (Fig. 3B). These results suggest that cholesterol excretion was reduced in the mice fed an FF/HS diet and may partly explain why the liver retained more cholesterol despite a reduced hepatic cholesterol synthesis. mRNA expression of genes involved in bile acid synthesis (*Cyp7a1*), bile acid excretion (*Abcb11*) (Gerloff et al., 1998), and cholesterol excretion (*Abcg5* and *Abcg8)* (Yu et al., 2002) was measured in liver. These were not significantly different between the two groups of mice suggesting that post-transcriptional regulation might be involved in bile acid excretion (Fig. 3C).

Hepatic uptake of cholesterol from plasma lipoproteins was increased in mice fed an FF/HS diet

Hepatocytes can acquire cholesterol from the uptake of plasma LDL and HDL. Therefore, we examined whether hepatic cholesterol uptake was altered in the liver of mice fed an FF/HS diet. Mice were injected with LDL labeled with ^{3}H-cholesteryl oleate, then hepatic content of ^{3}H-cholesteryl oleate was measured 1 h after injection. As shown in Fig. 4A, the hepatic content of ^{3}H-cholesterol from LDL was significantly increased in mice fed the FF/HS diet. mRNA expression of the LDL receptor was not changed compared to the mice fed a chow (Fig. 4B).

Next, the hepatic uptake of HDL-cholesterol was analyzed by injecting HDL labeled with ^{3}H-cholesteryl oleate. We observed that the hepatic content of ^{3}H-cholesteryl oleate from HDL was significantly increased in the mice fed an FF/HS diet (Fig. 4C). SR-BI is one of the major proteins responsible for the uptake of cholesterol from plasma HDL into the liver (Acton et al., 1996; Connelly and Williams, 2004). Liver mRNA expression level of SR-BI (*Scarb1*) was determined; it showed no change compared to the control group. However, SR-BI protein level was increased 3-fold in the mice fed with FF/HS diet compared to that in mice fed with a chow diet (Fig. 4F). CD36, also known as fatty acid translocase, is an integral membrane protein present on the surface of the cells. It binds various ligands including long-chain fatty acids, naive and oxidized LDL, and HDL (Brundert et al., 2011; Endemann et al., 1993; Febbraio and Silverstein, 2007). Both the mRNA expression and the protein level of CD36 were increased respectively by 1.8- and 1.6-fold in the liver of mice fed the FF/

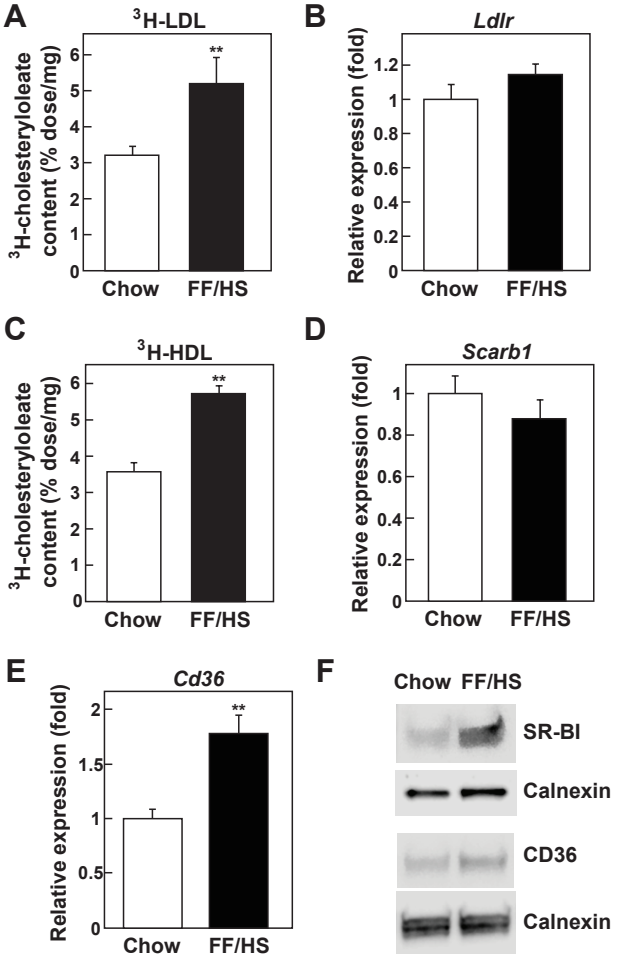

Fig. 4. *In vivo* hepatic uptake of LDL and HDL cholesterol in mice fed a chow or an FF/HS diet. Mice fed a chow or an FF/HS diet for 20 days were injected with ^{3}H-cholesteryl oleate-labeled LDL (A) or HDL (C). Uptake of ^{3}H-cholesteryl oleate from LDL (A) and HDL (C) was determined. Values represent the mean ± SE of 10 mice. mRNA expression of *Ldlr* (B), *Scarb1* (D), and *Cd36* (E) in the livers was determined by qPCR. Values represent the mean ± SE of 10 mice, relative to mice fed a chow diet, which is defined as 1. *Apoe* was used as the invariant control. The protein levels of SR-BI and CD36 were determined by immunoblotting. Equal amounts of membrane proteins individually prepared from 6 mice of the same group were pooled and used. Calnexin was used as a loading control (F). $**P < 0.01$ by Student's *t*-test.

HS diet compared to mice fed a chow diet (Figs. 4E and 4F). These results show that the hepatic uptake of plasma LDL- and HDL- cholesterol was increased in mice fed the FF/HS diet, which may further explain the increased hepatic cholesterol contents in these mice.

Higher fatty acid availability in hepatocytes facilitates cholesteryl ester formation and its incorporation into lipid droplets

Formation of cholesteryl esters, the storage form of cholesterol, was compared using primary hepatocytes isolated from

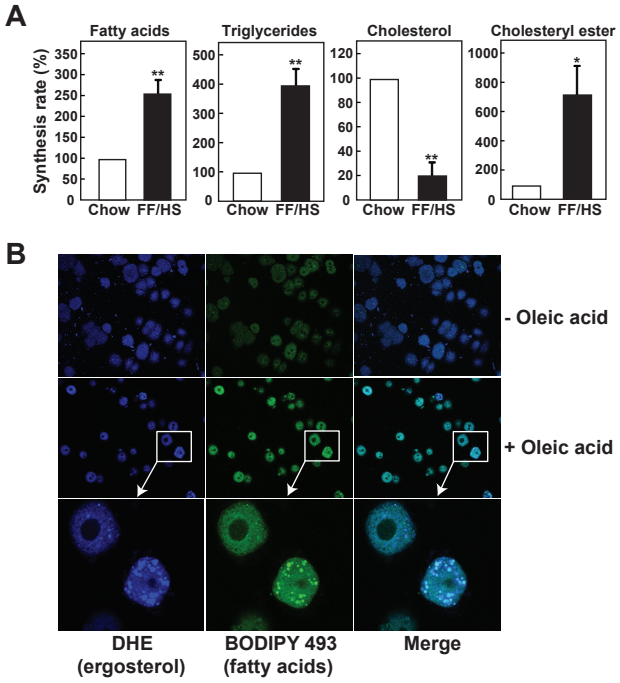

Fig. 5. Synthesis rates of cholesteryl esters and incorporation of cholesterol into lipid droplets in mouse primary hepatocytes. (A) Primary hepatocytes were isolated from mice fed a chow or an FF/HS diet. Cells were labeled with ^{14}C-acetate for 4 h, and the incorporation of ^{14}C-acetate into fatty acids, triglycerides, cholesterol, and cholesteryl esters was measured. The synthesis rates in hepatocytes isolated from mice fed a chow diet are defined as 100% and those in hepatocytes isolated from mice fed the FF/HS diet were compared. The values represent the mean ± SE of 3 experiments. $*P < 0.05$, $**P < 0.01$ by Student's *t*-test. (B) Primary hepatocytes isolated from mice fed a chow diet were incubated with DHE in the absence (-) or presence (+) of 20 μM oleic acid. DHE (blue) and triglycerides (BODIPY 493/503, green) were detected by confocal microscopy.

mice fed a chow or an FF/HS diet. Hepatocytes were incubated with ^{14}C-acetate, and its incorporation into fatty acids, triglycerides, cholesterol, and cholesteryl esters was measured (Fig. 5A). Consistent with the *in vivo* synthesis results, hepatocytes isolated from mice fed an FF/HS diet exhibit higher synthesis rates of fatty acids and triglycerides (Fig. 5A). Although cholesterol synthesis was lower in hepatocytes of the FF/HS-fed group, cholesteryl ester formation was higher compared to the chow-fed group (Fig. 5A). These results suggest that the conversion of cholesterol to cholesteryl esters were elevated in hepatocytes isolated from the FF/HS fed mice. This process may be driven by higher fatty acid availability from the upregulated *de novo* synthesis of fatty acids within the cells.

To examine whether fatty acids in hepatocytes can affect the localization of cholesterol in lipid droplets, primary hepatocytes were isolated from C57BL/6J mice and incubated with fluorescence-labeled DHE, a tracer of cholesterol, in either the presence or absence of oleic acid. BODIPY 493/503 was used to stain neutral lipid droplets (Fig. 5B). When the

cells were incubated with DHE in the presence of oleic acid, a strong fluorescence from DHE was detected within the cytosolic lipid droplets, while fluorescence from DHE was dispersed and weak in the absence of oleic acid (Fig. 5B). Results in Fig. 5 suggest that increased fatty acids in hepatocytes facilitates the incorporation of cholesterol into lipid droplets, mainly as cholesteryl esters. Increased cholesteryl ester formation and facilitated incorporation into lipid droplets may further explain the increased hepatic cholesterol content in mice with high *de novo* lipogenesis.

Hepatic VLDL secretion was increased in mice fed an FF/HS diet

Lipids stored in cytoplasmic lipid droplets are mobilized through lipid droplet-associated proteins for the lipidation of apoB to form VLDL (Ye et al., 2009; Zhang et al., 2017). To determine whether fatty liver induced by the FF/HS diet secretes more VLDL-triglyceride and -cholesterol, plasma triglyceride and cholesterol concentrations were measured at various time points after injection of tyloxapol, an inhibitor of lipoprotein lipase. Mice fed an FF/HS diet secreted more VLDL-triglyceride and -cholesterol into the plasma compared to the chow-fed group (Fig. 6A). The expression levels of genes that are essential for VLDL assembly, such as *Apob,* microsomal triglyceride transfer protein (*Mttp*), and phospholipid transfer protein (*Pltp*), were measured (Fig. 6B). The gene expression of ApoB, a key component of VLDL particles, was not significantly changed, while the expression of Mttp, a key player in the lipoprotein assembly, and Pltp, a protein involved in the lipidation and the secretion of VLDL (Okazaki et al., 2010; Yazdanyar and Jiang, 2012), were increased in mice fed an FF/HS diet (Fig. 6B). The expression of genes encoding proteins that affect VLDL-triglyceride hydrolysis through inhibition of lipoprotein lipase activity, such as *Angptl4* (Sukonina et al., 2006), *Angptl8* (Zhang, 2012), and *Apoc3* (Yao and Wang, 2012), were determined. The mRNA expression levels of *Angptl4* and *Angptl8* did not differ between the groups (Fig. 6C). *Apoc3* mRNA expression was slightly elevated in the liver of the mice fed the FF/HS diet (Supplementary Fig. S1), whereas plasma APO-CIII protein level showed no difference between groups (Fig. 6D). Even though more lipids were secreted as VLDL into the plasma of the mice fed an FF/HS diet, we did not observe an increase in plasma VLDL- or LDL-triglycerides and cholesterol most likely because of an intact LDL receptor function in C57BL/6J mice (Supplementary Fig. S2) (Horton et al., 1999).

Hepatic steatosis induced by FF/HS diet leads to severe hypercholesterolemia in *Ldlr*$^{-/-}$ mice

To determine the effects of an FF/HS diet on dyslipidemia, *Ldlr*$^{-/-}$ mice that exhibit a more human like lipoprotein profile as well as impaired uptake of ApoB-containing lipoproteins were used. Plasma concentrations of triglycerides and cholesterol and lipoprotein profiles were analyzed. Chow fed *Ldlr*$^{-/-}$ mice developed hypercholesterolemia with a moderate elevation of LDL cholesterol (Figs. 7A and 7B). *Ldlr*$^{-/-}$ mice fed an FF/HS diet showed increased hepatic triglyceride and cholesterol content (Fig. 7C) and exhibited higher concentrations of plasma cholesterol and triglycerides (Fig. 7A). Moreover, *Ldlr*$^{-/-}$ mice fed the FF/HS diet developed a dramatic increase in plasma VLDL- and LDL-cholesterol concentrations accompanied with higher VLDL-triglyceride levels (Fig. 7B). These results suggest that when the LDL receptor function is impaired, hepatic steatosis induced by FF/HS diet leads to severe dyslipidemia, characterized by hypercholesterolemia as well as hypertriglyceridemia.

DISCUSSION

SREBP-1c activation leads to hepatic triglyceride accumulation and hypertriglyceridemia in different animal models including hamsters fed a high sucrose diet (Moon et al., 2012), Tg-SREBP1a; *Ldlr*$^{-/-}$ mice (Horton et al., 1999), and *Ldlr*$^{-/-}$ mice treated with an LXR activator (T0901317) (Okazaki et al., 2010). These animal models also exhibit an elevation of hepatic cholesterol content and hypercholesterolemia,

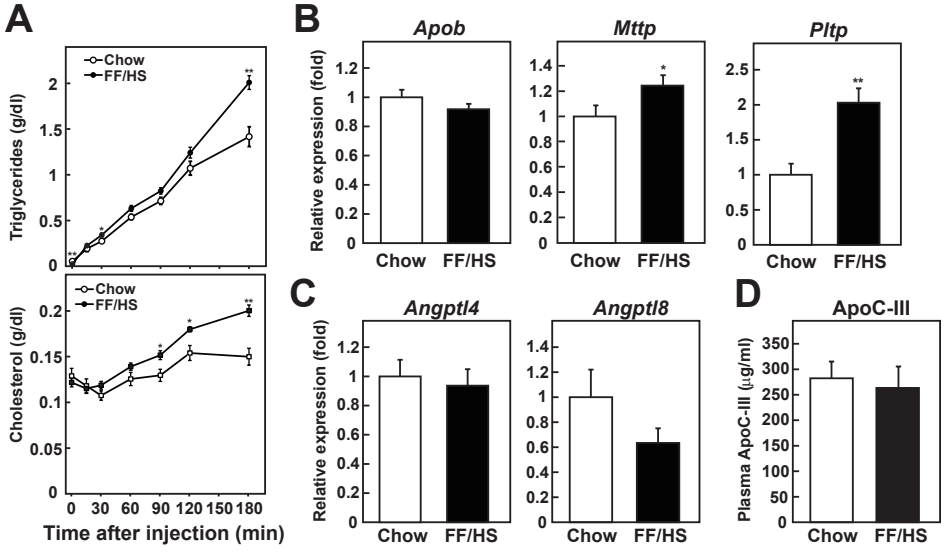

Fig. 6. *In vivo* VLDL secretion from livers of mice fed a chow diet or an FF/HS diet. (A) Mice were fed a chow or an FF/HS diet for 20 days and were then injected with tyloxapol. Blood was collected at the indicated time points, and concentrations of triglycerides and cholesterol were measured. Values represent the mean ± SE of 8 mice. mRNA expression of genes related to VLDL secretion (B) and triglyceride lipolysis (C) was measured by qPCR. (D) Plasma ApoC-III level was determined by ELISA. Each bar represents the mean ± SE of 10 mice. $*P < 0.05$, $**P < 0.01$ by Student's *t*-test.

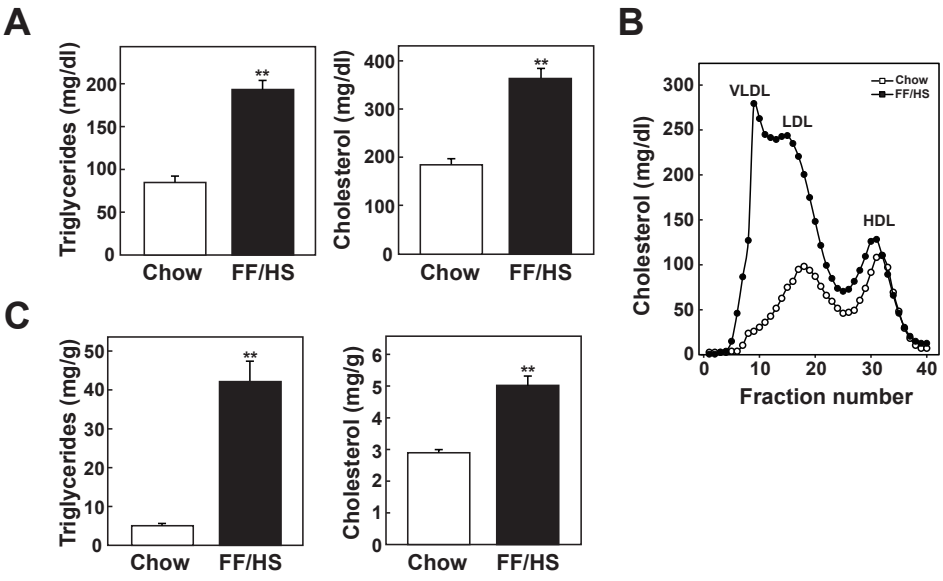

Fig. 7. Development of hypertriglyceridemia and hypercholesterolemia in *Ldlr*$^{-/-}$ mice fed an FF/HS diet. Male *Ldlr*$^{-/-}$ mice were fed a chow or an FF/HS diet for 20 days. Plasma concentrations of triglycerides and cholesterol (A) and lipoprotein profiles were determined (B). (C) Hepatic contents of triglycerides and cholesterol were measured. The data represent the mean ± SE of 8 mice. $^{**}P < 0.01$ by Student's *t*-test.

characterized by a substantial increase in VLDL-cholesterol levels. While SREBP-1c activation was the primary reason for hypertriglyceridemia, the exact mechanisms for hypercholesterolemia have not been fully investigated. In the present study, we determined how hepatic *de novo* lipogenesis influences hepatic cholesterol content and cholesterol metabolic pathways. Hepatic lipogenesis was induced in mice by feeding an FF/HS diet. In these mice, SREBP-1c was activated, which induced the expression of genes involved in fatty acid and triglyceride synthesis, and as a result, hepatic triglyceride concentrations increased. Cholesterol content was also elevated in the liver, although the mechanism was different from that of triglyceride accumulation. In contrast to fatty acid synthesis, cholesterol synthesis was suppressed. However, no significant change was observed in SREBP-2 activity or the expression of its target genes required for cholesterol synthesis. Nevertheless, the liver retained more cholesterol as a result of reduced excretion of bile acids and reduced excretion of cholesterol into feces. Concomitantly, the liver acquired more cholesterol from plasma LDL and HDL. The liver secreted more VLDL-TG and -cholesterol, which resulted in hypertriglyceridemia and hypercholesterolemia in *Ldlr*$^{-/-}$ mice.

The chow diet and the FF/HS diet used in this study were of different nature and it has been shown that dietary fiber contents might influence the rate of cholesterol excretion into the feces. We used diets with similar total dietary fiber contents for this study as the chow diet contains 17% fiber and the FF/HS diet contains 16.5% fiber. However, there is still a possibility that the difference in the nature of the diets might affect the cholesterol retention in the body.

The availability of fatty acids facilitates the conversion of cholesterol to cholesteryl esters (Xie et al., 2002), which could be readily incorporated into lipid droplets in hepatocytes, as presented in Fig. 5. Fatty acids not only drove cholesterol esterification, but also enhanced secretion of cholesteryl esters into the plasma (Xie et al., 2002). Acyl-coenzyme A: cholesterol acyltransferase 2 (ACAT2), the enzyme responsible for cholesteryl ester formation in the liver, prefers oleic acid as a substrate, and its activity is primarily regulated by the availability of fatty acid substrates (Xie et al., 2002). Upon activation of fatty acid synthesis by SREBP-1c in the liver, oleic acid (C18:1, n-9) is the fatty acid that is the most increased (Moon et al., 2014; Shimomura et al., 1998). Although ACAT2 mRNA expression levels were not significantly different between mice fed a chow and an FF/HS diet (Supplementary Fig. S1C), our findings suggest that the increased fatty acid availability from *de novo* synthesis in mice fed an FF/HS diet might drive cholesteryl ester formation (Fig. 5). This induced cholesterol incorporation into lipid droplets along with triglycerides accumulation in the hepatocytes.

Triglycerides and cholesteryl esters are packed into VLDL in hepatocytes. SREBP-1c increases PLTP expression as well as genes required for fatty acid synthesis. PLTP plays a major role in the incorporation of phospholipids to growing VLDLs to expand the particle and its lipidation, resulting in bigger VLDL particles rich in triglycerides (Okazaki et al., 2010; Yazdanyar and Jiang, 2012). The FF/HS diet induced PLTP expression as well as other targets of SREBP-1c, leading to an increased VLDL-triglyceride and -cholesterol secretion and subsequent development of hypercholesterolemia and hypertriglyceridemia in *Ldlr*$^{-/-}$ mice.

In humans, while fatty acids released from adipose tissue are the major source of fat in livers of individuals with NAFLD (Donnelly et al., 2005), *de novo* lipogenesis is also increased, and inhibition of lipogenesis through the inhibition of ACC markedly reduced hepatic fat accumulation (Kim et al., 2017; Lambert et al., 2014). High carbohydrate diets stimulate *de novo* lipogenesis and induce hypertriglyceridemia in humans (Chong et al., 2007; Hellerstein and Parks, 2000; Hudgins et al., 1996; Sanders et al., 2018). Findings in this study suggest that dietary cholesterol intake and cholesterol synthesis rates are only partially responsible for the hypercholesterolemia associated with NAFLD and that the control of fatty acid resources and fatty acid synthesis rates should also be considered for the management of NAFLD-associated hypercholesterolemia.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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AUTHOR CONTRIBUTIONS

J.M.B. and Y.A.M. conceived and performed experiments, analyzed the data, and wrote the manuscript.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

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