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Synergistic Interaction of Dietary Cholesterol and Dietary Fat in Inducing Experimental Steatohepatitis

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Abstract

The majority of patients with nonalcoholic fatty liver disease (NAFLD) have “simple steatosis,” which is defined by hepatic steatosis in the absence of substantial inflammation or fibrosis and is considered to be benign. However, 10%–30% of patients with NAFLD progress to fibrosing nonalcoholic steatohepatitis (NASH), which is characterized by varying degrees of hepatic inflammation and fibrosis, in addition to hepatic steatosis, and can lead to cirrhosis. The cause(s) of progression to fibrosing steatohepatitis are unclear. We aimed to test the relative contributions of dietary fat and dietary cholesterol and their interaction on the development of NASH. We assigned C57BL/6J mice to four diets for 30 weeks: control (4% fat and 0% cholesterol); high cholesterol (HC; 4% fat and 1% cholesterol); high fat (HF; 15% fat and 0% cholesterol); and high fat, high cholesterol (HFHC; 15% fat and 1% cholesterol). The HF and HC diets led to increased hepatic fat deposition with little inflammation and no fibrosis (i.e., simple hepatic steatosis). However, the HFHC diet led to significantly more profound hepatic steatosis, substantial inflammation, and perisinusoidal fibrosis (i.e., steatohepatitis), associated with adipose tissue inflammation and a reduction in plasma adiponectin levels. In addition, the HFHC diet led to other

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features of human NASH, including hypercholesterolemia and obesity. Hepatic and metabolic effects induced by dietary fat and cholesterol together were more than twice as great as the sum of the separate effects of each dietary component alone, demonstrating significant positive interaction.

Conclusion—Dietary fat and dietary cholesterol interact synergistically to induce the metabolic and hepatic features of NASH, whereas neither factor alone is sufficient to cause NASH in mice.

Nonalcoholic fatty liver disease (NAFLD) is the most-common liver disease in the United States, affecting 15%–46% of adults.^{1,2} The majority of patients with NAFLD have “simple steatosis” defined by excessive fat deposition within hepatocytes in the absence of substantial inflammation or fibrosis. This is generally considered to be a benign condition with little long-term harm to liver function.³ However, approximately 10%–30% of patients with NAFLD develop nonalcoholic steatohepatitis (NASH),² characterized by varying degrees of hepatic inflammation and fibrosis, in addition to hepatic steatosis. Steatohepatitis can progress to cirrhosis, liver failure, and hepatocellular carcinoma in a variable proportion of patients.^{3,4}

Although central obesity and insulin resistance (IR) have been clearly established as risk factors for fatty liver disease, the cause(s) of progressive steatohepatitis remain unclear. Consequently, it is currently unclear why the majority of patients with NAFLD related to obesity and IR have “benign” simple steatosis, whereas a small proportion develop progressive steatohepatitis or even cirrhosis.

Recent reports by our group and others suggest that dietary cholesterol is a critical factor in the development of experimental steatohepatitis in animal models.^{5–8} Human studies also support the hypothesis that dietary cholesterol plays a role in the development of steatohepatitis. In a large, nationally representative epidemiological study, we reported that dietary cholesterol consumption was independently associated with the development of cirrhosis.⁹ Finally, recent pilot clinical trials of ezetimibe, which inhibits intestinal cholesterol absorption, in humans with NASH have found improvements in hepatic inflammation and steatosis, although these studies were not randomized or controlled.^{10,11}

Not yet clarified is the unique contribution of dietary cholesterol separate from the contribution of dietary fat as well as the interaction between dietary fat and cholesterol in the development of steatohepatitis and its associated metabolic abnormalities. Our aims were to determine whether (1) high dietary fat can induce steatohepatitis in the absence of dietary cholesterol; (2) high dietary cholesterol can induce steatohepatitis in the absence of high dietary fat; and (3) high dietary fat together with high dietary cholesterol interact synergistically to induce steatohepatitis as well as the metabolic abnormalities associated with human NASH.

Materials and Methods

Animals and Experimental Design

Male C57BL/6J littermate mice were assigned to one of the following four diets for 30 weeks: (1) low-fat (4%, w/w) rodent chow with no cholesterol, henceforth called the

“control” diet (n = 8); (2) low-fat (4%), high-cholesterol (1%, w/w) diet, or the HC diet (n = 8); (3) high-fat (15%, w/w) diet with no cholesterol, or the HF diet (n = 8); and (4) high-fat (15%), high-cholesterol (1%) diet, or the HFHC diet (n = 8).

Mice were housed 4 per cage, with a 12-hour light/dark cycle and free access to food and water. Mice were euthanized 30 weeks after initiation of the experimental diets by cervical dislocation subsequent to isoflurane anesthesia. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Veterans Affairs Puget Sound Health Care System (Seattle, WA). In preliminary experiments, mice were also sacrificed at 8 and 12 weeks, as well as at 30 weeks, after initiation of the experimental diets to investigate the time course of induced hepatic histological changes.

Preparation of Experimental Diets

The low-fat, no-cholesterol rodent chow (i.e., the control diet) was purchased from Bio-Serv (Frenchtown, NJ). Cocoa butter, which contains approximately 60% saturated fat, was added by Bio-Serv into the control diet to increase the fat content from 4% in the control diet to 15% in the HF and HFHC diets. Cholesterol, purchased from Steraloids (Newport, RI), was purified in our laboratory by methanol recrystallization to remove oxysterol impurities, then sent to Bio-Serv for incorporation into the HC and HFHC diets. To avoid potential oxidation during the 30-week experiment, each diet was repackaged into weekly portions, flushed with nitrogen gas, vacuum-sealed, and stored at -70°C in the dark. Details of the composition of the diets are given in Table 1.

Hepatic Histology

Formalin-fixed liver sections were stained with hematoxylin and eosin, Masson’s trichrome, and Sirius Red. Histological steatosis, inflammation, and fibrosis were assessed semiquantitatively by a single, “blinded” liver pathologist (M.Y.), according to the scoring system proposed by Brunt et al. and recently modified by Kleiner et al.¹² In addition, the Sirius Red stain, which reacts specifically with collagen and does not stain other matrix proteins, was used for quantitative morphometric analyses to estimate the percentage of surface area staining positively for collagen in each liver. Under cross-polarizing light, collagen stained with Sirius Red appears bright red on a black background, thus facilitating morphometric analysis. Using a Nikon Eclipse microscope (Nikon Corporation, Tokyo, Japan) with a polarizing filter system, images of 10 random fields were taken ($\times 200$ original magnification; 0.26 mm^2 total area/image) in each liver, avoiding major blood vessels. ImageJ density software (National Institutes of Health, Bethesda, MD) was used to calculate the percent area of each image that stained positive for Sirius Red, and the average of 10 images per liver was calculated.

Hepatic Lipid Analysis

Hepatic lipids were extracted from frozen liver using Folch’s method.¹³ Neutral lipid fractions were prepared by solid-phase extraction on Bond Elut SI cartridges (Varian, Inc., Walnut Creek, CA), and triglycerides (TGs), diglycerides, cholesterol esters, and free cholesterol were then separated and quantified by normal-phase high-performance liquid chromatography/evaporative light-scattering detection (HPLC/ELSD). Free fatty acids were

derivatized by boron trifluoride/methanol, and methyl esters of fatty acids were separated by gas chromatography, using a 60-m HP-INNOWax capillary column (Agilent Technologies, Inc., Santa Clara, CA).

Hepatic and Adipose Tissue Real-Time Quantitative Reverse-Transcription Polymerase Chain Reaction

Total RNA was isolated from whole liver and epididymal (i.e., intra-abdominal) adipose tissue using RNeasy Mini Columns (Qiagen, Valencia, CA) and reverse transcribed to complementary DNA. Quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) was performed using the ABI 7500 sequence detection system (Applied Biosystems, Foster City, CA). Assays were performed in triplicate, and results are expressed as relative gene expression normalized to expression levels of housekeeping genes (β -actin for liver and glyceraldehyde 3-phosphate dehydrogenase for adipose tissue).

Other Measurements

Blood specimens were collected immediately before sacrifice after a 4-hour fast and tested for plasma cholesterol, TGs, alanine aminotransferase (ALT), and glucose (Phoenix Central Laboratory, Everett, WA) using standard methodology. Plasma insulin was assayed using a commercially available kit (Linco Research, Inc., St. Charles, MO). Plasma high-molecular-weight (HMW) adiponectin, which mediates most of the metabolic activities of circulating adiponectin,¹⁴ was measured using a sandwich enzyme-linked immunosorbent assay kit from Alpco Diagnostics (Salem, NH). Plasma tumor necrosis factor alpha (TNF- α), interleukin (IL)-1b, and IL-6 levels were measured using a cytokine multiplex assay (Bio-Plex 200 System; Bio-Rad, Hercules, CA). A complete fecal collection was performed over a 48-hour period while the mice were individually housed. Fecal lipids were extracted using Folch's technique and analyzed by HPLC/ELSD. Mice were weighed at baseline and 10, 20, and 30 weeks. Food consumption was measured and averaged over three 4-day periods between 0–10, 10–20, 20–30, and 30–40 weeks (i.e., a total of twelve 4-day periods).

Statistical Analysis

Results were expressed as proportions or means $\pm$ standard deviation (SD). Mean values of normally distributed continuous variables were compared using a *t* test; Wilcoxon's nonparametric test was used for non-normally distributed continuous variables. The chi-square test was used to compare categorical variables. A two-tailed *P* value <0.05 was considered statistically significant. We performed formal statistics for two comparisons: the HFHC versus the HF group and the HC versus the control group. We also tested for statistical interaction between dietary fat and dietary cholesterol by including an interaction term (i.e., dietary cholesterol $\times$ dietary fat) as well as the separate variables for dietary fat and dietary cholesterol in multivariate linear regression models of selected outcomes.

Results

HFHC Diet Causes Greater Weight Gain Than the HF or HC Diets

The four dietary groups had similar baseline weight (mean = 30.4 ± 1.1 g). By the end of the 30-week experiment, body weight did not change in mice on the control diet or HC diet

(Table 2; Fig. 1). In contrast, mice on the HF diet gained 13% in weight, compared with their baseline, whereas mice on the HFHC diet gained significantly more weight, at 32% ($P < 0.05$). There was significant statistical interaction between dietary fat and dietary cholesterol in inducing weight gain (Table 2), demonstrating that the weight gain observed by the presence of both high fat and high cholesterol in the HFHC diet was significantly greater than what would be expected by adding the individual effects of high fat and high cholesterol.

The greater weight gain of the mice on the HFHC diet could not be attributed to greater caloric dietary intake. In fact, mice on an HFHC diet consumed significantly ($P < 0.05$) less food than mice on the isocaloric HF diet throughout the 30-week experiment (Fig. 1) and yet gained significantly more weight, suggesting lower energy expenditure in mice on the HFHC diet. Excretion of fecal free fatty acids (FFAs) was slightly lower in the HFHC than in the HF group (Table 2), suggesting more-efficient intestinal absorption of fatty acids in the HFHC group than the HF group, which may also have contributed to greater weight gain.

HFHC Diet Causes Greater Hepatic Lipid Accumulation Than the HF or HC Diets

Liver weight was only slightly greater on the HF diet (1.7 ± 0.8 g) and the HC diet (1.6 ± 0.5 g) than on the control diet (1.2 ± 0.1 g). However, liver weight was almost three times greater on the HFHC diet (3.4 ± 0.1 g) than on the control diet (Table 2; Fig. 2). Liver weight/body weight ratio was only significantly increased on the HFHC diet ($8.6\% \pm 1.7\%$) and not on the HF ($4.7\% \pm 0.8\%$) or HC ($5.0\% \pm 1.0\%$) diets, relative to the control diet ($4.2\% \pm 0.3\%$). There was significant positive interaction between dietary fat and dietary cholesterol in inducing hepatic lipid deposition and increasing liver weight (Table 2), showing that their combined effect was much greater than the sum of their individual effects. Analysis of hepatic lipid composition confirmed that the increased liver weight was the result of a profound deposition of TGs and, to a lesser extent, cholesterol esters in the liver (Table 2; Fig. 2). Unesterified (i.e., “free”) cholesterol level and diglyceride levels were significantly increased only in the HFHC group. FFA levels were not substantially different between the four dietary groups.

HFHC Diet Increases Plasma Levels of Liver Enzymes and Fasting Lipids and Decreases HMW Adiponectin

Serum ALT level, a marker of hepatic necroinflammation, was normal in the control group (33 ± 22 U/L), very mildly elevated in the HF (88 ± 83 U/L) and HC (51 ± 34 U/L) groups, and almost 10-fold elevated in the HFHC group (319 ± 111 U/L) (Table 2; Fig. 3). Plasma cholesterol level was elevated only in the HFHC group (262 ± 66 mg/dL). There was significant statistical interaction between dietary fat and dietary cholesterol in causing elevated plasma cholesterol and ALT levels. Plasma TG and glucose levels were similar across all four dietary groups. Serum insulin levels were elevated strikingly on the HF, HC, and, especially, the HFHC diets, relative to the control group. HMW adiponectin levels were significantly reduced in the HFHC group, relative to all other groups. HMW adiponectin levels were also reduced in the HC, relative to the control, group.

Hepatic Messenger RNA Studies Suggest That Dietary Cholesterol Exacerbates Hepatic Lipid Loading by Reducing Bile Acid Synthesis, Very-Low-Density Lipoprotein Synthesis, and Beta-Oxidation of Fatty Acids

Compared to the HF-fed mice, those on the HFHC diet overexpressed cholesterol export genes (e.g., ATP-binding cassette transporter G5 [*abcg5* and *abcg8*]) and underexpressed cholesterol synthesis genes (e.g., 3-hydroxy-3-methylglutaryl-coenzyme A [*HmgCoA*]-reductase and *HmgCoA-synthase*) and low-density lipoprotein receptor, the free cholesterol uptake transporter, as expected, given the high cholesterol content of their diet (Fig. 4A). Also, compared to HF-fed mice, those fed an HFHC diet had lower expression of *Fas* (rate limiting for *de novo* synthesis of fatty acids by the liver), suggesting an attempt to reduce the amount of fat synthesized and deposited in the liver. However, decreased expression of genes related to bile acid synthesis (e.g., cytochrome P450 [*cyp7a1* and *cyp27a1*]) was observed in mice fed an HFHC diet, relative to HF-fed mice. This suggests reduced biotransformation of cholesterol to bile acids, which represents the key mechanism for the excretion of excess cholesterol. Interestingly, the expression of acylCo:cholesterol acyltransferase 2 (*Acat2*) (rate limiting for cholesterol esterification) was reduced, suggesting a mechanism for the accumulation of potentially toxic free cholesterol. Also, HFHC-fed mice showed lower expression of microsomal TG transfer protein (*Mttp*), which is rate limiting for the synthesis of very-low-density lipoprotein (VLDL) and the export of lipids from the liver. In addition, peroxisome proliferator receptor alpha (*Ppara*) and carnitine palmitoyl transferase 1 alpha (*Cpt-1a*), genes related to the beta-oxidation of fatty acids, were underexpressed in HFHC-fed mice, relative to HF-fed mice, suggesting the failure of HFHC-fed mice to up-regulate fatty-acid beta-oxidation as a means of removing excess hepatic fatty acids. Key nuclear transcription factors that regulate hepatic cholesterol, lipid, carbohydrate, and bile acid metabolism (e.g., sterol regulatory binding protein-1, liver X receptor, farnesoid X receptor, and carbohydrate response element-binding protein) were all underexpressed in HFHC-fed, compared to HF-fed, mice. In summary, the addition of dietary cholesterol caused changes in the following pathways, which could have further contributed to the lipid loading of the liver: reduced bile-acid synthesis, reduced VLDL synthesis, and reduced beta-oxidation of fatty acids.

Qualitatively similar differences in messenger RNA (mRNA) gene expression were observed, when comparing the HC-fed mice to the control-fed mice and the HFHC-fed mice to the HF-fed mice (described above; Fig. 4A). Therefore, the addition of dietary cholesterol seemed to cause consistent changes in gene expression, whether it was added to the control diet or to the HF diet.

Hepatic gene-expression studies were also performed at 4 weeks after the introduction of the experimental diets and were found to have very similar, but slightly less pronounced, patterns to those described at 30 weeks (data not shown).

HFHC Diet Increases Adipose Tissue Inflammation

Macrophage gene expression (EGG-like module-containing mucin-like hormone receptor-like 1 [*F4/80*]), monocyte chemotactic factor genes (monocyte chemoattractant protein 1 and serum amyloid), and gene expression for *Tnfa*, a proinflammatory cytokine produced by

activated macrophages, were all significantly higher only in the HFHC-fed mice, suggestive of increased intra-abdominal adipose tissue inflammation in this group of mice (Fig. 4B). The expression of these genes was not induced by dietary fat or dietary cholesterol alone, demonstrating significant positive interaction between dietary fat and cholesterol in inducing adipose tissue inflammation.

HFHC Diet Causes Histological Fibrosing Steatohepatitis

Mice on the control diet developed no appreciable histological hepatic steatosis, inflammation, or fibrosis during the 30-week experimental period (Table 3; Fig. 5). Mice on the HF and HC diets developed mild to moderate, hepatic, macrovesicular steatosis (grade 2) in a predominant zone 3 distribution, with an associated mild inflammatory infiltrate (grade 1), but no histological fibrosis. In contrast, mice on the HFHC diet developed severe, zone 3, macrovesicular steatosis (grade 3) associated with inflammatory foci (grade 2) and perisinusoidal, “chickenwire” fibrosis (i.e., “fibrosing steatohepatitis”). This presence of fibrosing steatohepatitis was evident both by Masson’s trichrome stain and by Sirius Red stain (Fig. 5 and Supporting Figs. 1 and 2). Quantitative morphometric measures of Sirius Red staining confirmed the pathologist’s semiquantitative histological assessment: Sirius Red staining was substantially elevated (1.54%) only in the HFHC group, relative to the control (0.50%), HF (0.65%), and HC (0.88%) groups ($P < 0.05$ for each comparison), which stained primarily for collagen associated with minor blood vessels (Table 3).

Relative to mice on a control diet, mice fed an HFHC diet had significantly ($P < 0.05$) higher expression of genes known to be involved in hepatic fibrogenesis, notably procollagen Ia1 (8.5-fold), tissue inhibitor of metalloproteinase-1 (11.2-fold), matrix metalloproteinase (MMP)-2 (2.5-fold), and MMP-13 (5.8-fold); expression of these genes was either not elevated or elevated in a lower, non-significant degree in mice on an HC or HF diet.

Time Course of Histological Changes Induced by the HFHC Diet

Hepatic steatosis and inflammation were well established when mice were sacrificed at 8 and 12 weeks after initiation of the HFHC diet and were associated with elevated ALT, obesity, and IR (Supporting Table 1; Supporting Fig. 3). However, there was no fibrosis, either by Sirius Red or Masson’s trichrome stains, at 8 or 12 weeks. Instead, fibrosis and fibrosing steatohepatitis developed with longer exposure to the HFHC diet for 30 weeks.

Discussion

Human NASH is characterized by hepatic steatosis, with variable degrees of inflammation and fibrosis occurring in a typical metabolic phenotype of central obesity, IR, hypoadiponectinemia, and hyperlipidemia. Our results suggest that dietary fat and dietary cholesterol strongly interact in the development of both the hepatic histological abnormalities of NASH and its associated metabolic abnormalities. Only mice on the HFHC diet developed fibrosing steatohepatitis, suggesting that both dietary cholesterol and dietary fat are necessary for the development of steatohepatitis. Also, only mice on the HFHC diet developed hypercholesterolemia, elevated plasma ALT, and adipose tissue inflammation. Profound positive interactions between dietary cholesterol and dietary fat were observed in

their effects on body weight, liver weight, liver weight/body weight ratio, hepatic lipid concentration, and plasma ALT and cholesterol levels. The effects of dietary fat and cholesterol together on these parameters were more than two times greater than the sum of the effects observed with high dietary fat alone or high dietary cholesterol alone. This suggests that dietary fat and dietary cholesterol interact synergistically to induce the metabolic and hepatic features of NASH, whereas neither factor alone is sufficient to cause NASH.

We showed that in the setting of an HF diet, the addition of dietary cholesterol causes adipose tissue inflammation, resulting in a reduction of plasma HMW adiponectin levels. Impaired adiponectin production and signaling have been implicated in the development of NAFLD/NASH in mice and humans.¹⁴ Adiponectin regulates whole-body lipid partitioning, providing signals that convey TGs to adipose tissue, thus sparing the liver, as well as other sites, of ectopic fat deposition.¹⁵ Furthermore, adiponectin reduces inflammation, stimulating the secretion of anti-inflammatory cytokines (e.g., IL-10), blocking nuclear factor kappa light-chain enhancer of activated B cell activation, and inhibiting the release of TNF- α , IL-6, and chemokines.¹⁶ Finally, adiponectin protects against Fas-mediated hepatocyte death¹⁷ and reduces fibrogenesis, the latter mediated, at least in part, through a direct action on hepatic stellate cells.¹⁸ Therefore, the reduction in plasma adiponectin levels induced by dietary cholesterol and fat likely represents a major mechanism underlying the development of NASH that we observed in mice fed an HFHC diet, contributing to the development of all three major histological features of NASH (i.e., stasis, inflammation, and fibrosis).

Additional mechanisms by which dietary cholesterol might promote hepatic inflammation and fibrosis are also possible. The cytotoxicity of free cholesterol is well established.¹⁹ Potential mechanisms include sensitization of liver mitochondria to cytokine-mediated injury²⁰ and endoplasmic reticulum stress-mediated apoptosis.²¹ In rabbits, dietary cholesterol has been suggested to cause stellate cell activation, leading to perisinusoidal fibrosis.²² Finally, the induction of adipose tissue inflammation by dietary cholesterol, demonstrated in our experiments in the setting of high dietary fat, may cause changes in the secretion of adipokines other than adiponectin, such as leptin, IL-6, and TNF- α , which have also been implicated in human steatohepatitis (although we did not find elevated plasma TNF- α or IL-6 levels in HFHC mice; data not shown).²³

Hepatic mRNA expression studies highlighted three potential mechanisms by which increased dietary cholesterol may lead to increased hepatic steatosis: reduced beta-oxidation of fatty acids (e.g., *Ppara* and *Cpt-1a*); VLDL synthesis (e.g., *Mtp*); and bile-acid synthesis (e.g., *Cyp7a1*). Our results are consistent with previous studies suggesting that PPAR- α functions as a sensor for fatty acids and ineffective PPAR- α sensing can lead to reduced energy burning, resulting in hepatic steatosis and steatohepatitis.²⁴ Assembly of VLDL in the liver represents the main mechanism of exporting TGs and cholesterol from the liver into the bloodstream. Biotransformation of cholesterol into bile acids and excretion into bile represents a mechanism of eliminating cholesterol from the body. We recently reported that dietary cholesterol also led to a reduction in hepatic bile-acid synthesis in a different mouse model of NASH, the *foz/foz* mouse.⁸ Furthermore, in the current study, we found that

profound steatosis and elevated transaminases in all mice and fibrosing steatohepatitis “in a subset.”⁶ The steatohepatitis was partially reversed by treatment with ezetimibe, ⁶ which inhibits intestinal cholesterol absorption. However, this study did not distinguish the unique contributions of dietary fat versus dietary cholesterol on the development of fibrosing steatohepatitis. We have recently shown that the addition of dietary cholesterol to an HF, high-carbohydrate, “diabetogenic diet” led to increased hepatic steatosis, inflammation, and fibrosis in low-density lipoprotein receptor-deficient mice.⁷ We have also shown that increasing dietary cholesterol exacerbated hepatic accumulation of free cholesterol, hepatocyte injury, macrophage recruitment, and liver fibrosis in Alms 1 mutant (*foz/foz*) mice.⁸

Human data are also emerging to support a role for dietary cholesterol in the development of progressive NASH or cirrhosis. In a large, nationally representative epidemiological study, we reported that dietary cholesterol consumption was independently associated with the development of cirrhosis.⁹ Recent pilot clinical trials of ezetimibe in humans with NASH have found improvements in hepatic inflammation and steatosis, although these studies were not randomized or controlled.^{10,11} We believe that sufficient animal and human preliminary data are available to justify a randomized, controlled trial aiming to determine the effect of reducing cholesterol intake or inhibiting intestinal cholesterol absorption on the progression of NASH.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Abc	ATP-binding cassette transporter
Acat2	acylCo:cholesterol acyltransferase 2
ALT	alanine aminotransferase
Cpt-1a	carnitine palmitoyl transferase 1 alpha
Cyp	cytochrome P450
ELSD	evaporative light-scattering detection
FFAs	free fatty acids
HF	high-fat diet
HC	high-cholesterol diet

HFHC	high-fat and high-cholesterol diet
Hmg	3-hydroxy-3-methylglutaryl-coenzyme A
HMW	high molecular weight
HPLC	high-performance liquid chromatography
IL	interleukin
IR	insulin resistance
KO	knockout
MMP	matrix metalloproteinases
mRNA	messenger RNA
Mttp	microsomal triglyceride transfer protein
NAFLD	nonalcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
Pparaα	peroxisome proliferator receptor α
RT-PCR	reverse-transcription polymerase chain reaction
SD	standard deviation
TNF-α	tumor necrosis factor alpha
VLDL	very-low-density lipoprotein

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Fig. 1.

Dietary consumption and percent body-weight change during the 30-week experiment. Mice on the HFHC diet consumed fewer calories, but gained more weight, than mice on the isocaloric HF diet. There was no difference between mice on the HC diet and mice on the control diet in the amount of food consumed or in weight change during the experiment. Therefore, the addition of dietary cholesterol only caused weight gain in the presence of increased dietary fat.

Fig. 2.

Comparison* of experimental diets with respect to liver weight and lipid composition. *We calculated *P* values only for two comparisons: the HFHC versus the HF group and the HC versus the control group. Only *P* values < 0.05 are shown.

A) Plasma Laboratory Tests

B) Fecal Lipids

Fig. 3.

Comparison* of experimental diets with respect to (A) plasma laboratory tests and (B) fecal lipid composition. *We calculated *P* values only for two comparisons: the HFHC versus the HF group and the HC versus the control group. Only *P* values < 0.05 are shown.

Fig. 4. Comparison* of experimental diets with respect to (A) liver mRNA expression and (B) intra-abdominal adipose tissue mRNA expression. *We calculated *P* values only for two comparisons: the HFHC versus the HF group and the HC versus the control group. Only *P* values <0.05 are shown.

Fig. 5.

Mouse liver sections obtained after 30 weeks on each of the four experimental diets (control, HC, HF, HFHC) and stained with hematoxylin and eosin, Sirius Red (viewed with normal or polarized light), and Masson's trichrome. Sections demonstrate the following histological features: (A) control diet: healthy liver histology; (B) HC diet: mild to moderate steatosis with little inflammatory infiltrate and no fibrosis (i.e., simple steatosis); (C) HF diet: mild to moderate steatosis with little inflammatory infiltrate and no fibrosis (i.e., simple steatosis); and (D) HFHC diet: severe steatosis with substantial inflammatory infiltrate and perisinusoidal, "chickenwire" fibrosis consistent with NASH.

Table 1

Composition of Experimental Diets

	Control	HC	HF	HFHC
Protein, g/100 g	13	13	13	13
Carbohydrate, g/100 g	69	69	56	56
Fat, g/100 g	4	4	15	15
Cholesterol, g/100 g	0	1	0	1
Fatty acids, g/100 g				
C14:0	0	0	0.013	0.013
C16:0	0.42	0.42	3.6	3.6
C16:1	0	0	0.03	0.03
C18:0	0.18	0.18	4.5	4.5
C18:1	0.9	0.9	4.8	4.8
C18:2	2.0	2.0	1.4	1.4
C18:3	0.3	0.3	0.1	0.1
Total saturated	0.63	0.63	8.2	8.2
Total monounsaturated	0.91	0.91	4.8	4.8
Total polyunsaturated	2.31	2.31	1.5	1.5
Protein, kcal/g	0.5	0.5	0.5	0.5
Carbohydrate, kcal/g	2.8	2.8	2.3	2.3
Fat, kcal/g	0.4	0.4	1.4	1.4
Total kcal/g	3.6	3.6	4.2	4.2

Table 2

Body and Liver Weights, Hepatic and Fecal Lipid Composition, and Plasma Levels After 30 Weeks on Experimental Diets

Diet	Control	HC	HF	HFHC	Test of Interaction <i>P</i> Value [‡]
Body weight gain, %	-3.0 ± 1.0	0.4 ± 0.1	13 ± 4 [*]	32 ± 7 [*]	0.03
Liver weight, g	1.2 ± 0.1	1.6 ± 0.5	1.7 ± 0.8 [*]	3.4 ± 0.1 [*]	0.02
Liver weight/body weight, %	4.2 ± 0.3	5.0 ± 1.0	4.7 ± 1.3 [*]	8.6 ± 1.7 [*]	0.02
Hepatic lipids, mg/g					
TG	12.5 ± 3.9	69 ± 76	97 ± 81 [*]	235 ± 75 [*]	0.04
Diacylglyceride	1.3 ± 0.7	1.5 ± 0.7	1.2 ± 0.2 [*]	3.0 ± 1.3 [*]	0.02
Cholesterol ester	0.5 ± 0.4 [‡]	38 ± 26 [‡]	0.8 ± 0.6 [*]	108 ± 38 [*]	<0.001
Cholesterol	0.41 ± 0.31	0.59 ± 0.23	0.48 ± 0.23 [*]	1.22 ± 0.6 [*]	0.09
FFA	2.5 ± 0.5	3.0 ± 0.8	3.1 ± 0.6	3.7 ± 1.5	0.9
Plasma levels (fasting)					
ALT, U/L	33 ± 22	51 ± 34	88 ± 83 [*]	319 ± 111 [*]	0.01
Cholesterol, mg/dL	61 ± 26	87 ± 42	86 ± 49 [*]	262 ± 66 [*]	<0.001
TG, mg/dL	37 ± 22	29 ± 5	44 ± 12	35 ± 14	0.8
Glucose, mg/dL	262 ± 57	256 ± 57	239 ± 35 [*]	290 ± 44 [*]	0.06
Insulin, ng/mL	0.66 ± 0.71	2.57 ± 2.59	2.78 ± 1.77	3.26 ± 2.49	0.4
Adiponectin, µg/mL	6.6 ± 1.4 [‡]	4.4 ± 1.4 [‡]	5.2 ± 1.4 [*]	3.2 ± 0.8 [*]	0.8
Fecal lipids, mg/24 hours					
Cholesterol	0.02 ± 0.03 [‡]	3.4 ± 0.8 [‡]	0.2 ± 0.2 [*]	3.9 ± 1.8 [*]	0.6
FFAs	0.7 ± 0.2 [‡]	0.4 ± 0.1 [‡]	5.3 ± 0.3 [*]	4.0 ± 0.9 [*]	0.02

Data are presented as mean ± SD.

^{*} Indicates a comparison between HFHC versus HF that is statistically significant, with *P* < 0.05.[‡] Indicates a comparison between HC versus control that is statistically significant, with *P* < 0.05.[‡] The test of interaction is statistically significant (<0.05) if the effect observed in the presence of both dietary cholesterol and dietary fat (i.e., in the HFHC diet) is significantly different than what would be expected by adding the individual effects of dietary cholesterol and dietary fat.

Table 3

Effects of the Experimental Diets on Hepatic Histology at 30 Weeks

Diet (N = 8 animals for each)	Control	HC	HF	HFHC
Steatosis (0–3) *	0	2	2	3
Lobular inflammation (0–3) †	0	1	1	2
Fibrosis (0–4) ‡	0	0	0	1a
Hepatic Sirius Red staining, %	0.50 ± 0.21	0.88 ± 0.63	0.65 ± 0.52	1.54 ± 1.11

Median values are reported for steatosis, inflammation, and fibrosis and mean ± SD for percent Sirius Red staining.

* Steatosis is graded as: <5% (0); 5%–33% (1); 34%–66% (2); and >66% (3).

† Lobular Inflammation combines mononuclear, fat granulomas, and polymorphonuclear leucocytes and is graded as: none (0); <2 per ×200 magnification (1); 2–4 per ×200 magnification (2); and >4 per ×2,000 magnification (3).

‡ Fibrosis is staged as: none (0); perisinusoidal (1a); periportal (1b); periportal and perisinusoidal (2); bridging fibrosis (3); and cirrhosis (4).