Dietary Cholesterol Exacerbates Hepatic Steatosis and Inflammation in Obese LDL Receptor-Deficient Mice


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Running title: Dietary cholesterol and steatohepatitis
Abstract:
Non-alcoholic fatty liver disease (NAFLD), the hepatic manifestation of the metabolic syndrome, can progress to steatohepatitis (NASH) and advanced liver disease. Mechanisms that underlie this progression remain poorly understood, partly due to lack of good animal models that resemble human NASH. We previously showed that several metabolic syndrome features that develop in LDL receptor-deficient (LDLR-/-) mice fed a diabetogenic diet are worsened by dietary cholesterol. To test whether dietary cholesterol can alter the hepatic phenotype in the metabolic syndrome, we fed LDLR-/- mice a high fat, high carbohydrate diabetogenic diet, without (DD) or with (DDC) added cholesterol. Both groups of mice developed obesity and insulin resistance. Hyperinsulinemia, dyslipidemia, hepatic triglyceride and ALT elevations were greater with DDC. Livers of DD-fed mice showed histological changes resembling NAFLD, including steatosis and modest fibrotic changes; however, DDC-fed animals developed micro- and macrovesicular steatosis, inflammatory cell foci and fibrosis resembling human NASH. Dietary cholesterol also exacerbated hepatic macrophage infiltration, apoptosis and oxidative stress. Thus, LDLR-/- mice fed diabetogenic diets may be useful models for studying human NASH. Dietary cholesterol appears to confer a second “hit” that results in a distinct hepatic phenotype characterized by increased inflammation and oxidative stress.

Key words: Fatty liver, metabolic syndrome, oxysterols, apoptosis, oxidative stress, HODE, HETE, oxidized fatty acids
Introduction:

The obesity epidemic has led to a dramatic increase in the incidence of the metabolic syndrome, insulin resistance and type 2 diabetes. Nonalcoholic fatty liver disease (NAFLD) is a liver disorder strongly associated with obesity, type 2 diabetes and insulin resistance (1). The spectrum of changes in the liver in NAFLD ranges from simple, non-inflammatory triglyceride accumulation in hepatocytes ("simple" steatosis or fatty liver) to steatosis with inflammation and fibrosis (steatohepatitis), occasionally progressing to cirrhosis, end-stage liver disease and hepatocellular carcinoma (2). Thus, NAFLD has emerged as a substantial public health concern and is now considered to be hepatic manifestation of the metabolic syndrome (3).

Triglyceride accumulation in hepatocytes increases vulnerability of the liver to secondary insults through effects of cytokines or oxidative stress (2). While triglyceride accumulation is believed to occur initially (the "first hit"), it is postulated that progression to more advanced stages of NAFLD, including inflammation, fibrosis (NASH) and cirrhosis requires a "second hit" superimposed upon hepatic steatosis. This second hit may include genetic susceptibility, dietary factors or environmental stressors. However, the precise molecular signals that trigger this change have not yet been identified.

Several dietary and genetic mouse models have been used to study the pathogenesis of NAFLD. Of these, the methionine-choline deficient (MCD) diet has been used widely to induce a dietary model of NASH in rodents (4). This diet rapidly leads to intra-hepatic lipid accumulation with cell injury and cell death due to impaired synthesis of phosphotidylcholine, an essential component of lipoprotein phospholipid (4). However, this dietary model results in decreased plasma glucose and insulin levels, improved insulin sensitivity and weight loss and therefore a metabolic phenotype that is very different from that seen in metabolic syndrome in humans. This greatly limits extrapolation of findings in the MCD model to human steatohepatitis. Additional rodent models of NAFLD have involved dietary manipulations in genetically mutant mice such as leptin deficient (ob/ob), leptin receptor deficient (db/db) (5), and apoE2 knock-in
mice (6). In these models, high fat diet feeding induces insulin resistance and triglyceride accumulation in the liver. However, these models lack inflammation and/or fibrosis that are required to fulfill the criteria of NASH (4). Models in which caloric overload by intra-gastric and enteral feeding is used to induce hepatic steatosis and steatohepatitis (7) show significant variability in the features of NAFLD observed.

Cholesterol feeding can induce several features of the metabolic syndrome, such as dyslipidemia and insulin resistance (8, 9). In animal models, dietary cholesterol appears to be an important risk factor for hepatic steatosis and progression to steatohepatitis (10, 11). However, wide variations in cholesterol content in diets (0.2-2%) and lack of development of steatosis and/or obesity limit extrapolation of these animal models to human NASH (8, 10-12).

The LDL receptor deficient (LDLR-/-) mouse develops many features of the metabolic syndrome when fed a diet rich in saturated fat and refined carbohydrates ("diabetogenic" diet). These include obesity, insulin resistance and dyslipidemia, as well as local (adipose tissue), systemic inflammation and atherosclerosis (9, 13). We previously have shown that addition of a small amount of added cholesterol (0.15%) to a diet rich in saturated fat and refined carbohydrate increases insulin resistance, adipose tissue inflammation, chronic systemic inflammation, and atherosclerosis in LDLR-/- mice (9). In the present study we have used LDLR-/- mice fed diabetogenic diets without or with added cholesterol to investigate the effects of dietary cholesterol on the hepatic phenotype in the metabolic syndrome. We show that the LDLR-/- mouse is an attractive rodent model to study changes occurring in the liver in NAFLD, and that dietary cholesterol plays an important role in hepatic fat accumulation, inflammation and fibrosis characteristic of NASH. Importantly, an increase in lipid peroxidation products in the liver suggests that oxidative stress is involved in the pathogenesis of steatohepatitis in this model.

**Methods:**
Animals and diets: Adult (10 week old) male LDL receptor-deficient mice on a C57BL/6 background were fed rodent chow, a “diabetogenic” high fat diet (DD, provides 35.5% carbohydrates and 36.6% as fat, F1850, Bio-serv, Frenchtown, NJ) or a diabetogenic diet with 0.15% added cholesterol (DDC, F4997, Bio-serv, Frenchtown, NJ). Details of these diets have been published previously (9). Diets were free of added antioxidants and were changed approximately every 3 days. Diets were stored in a similar fashion to regular chow diet without any added precautions. Both high fat diets were stored in small plastic containers and stored at -70°C until processing for measurement of oxysterols. Mice were maintained in a temperature and light-controlled facility and received the diets ad libitum for a total of 24 weeks. Blood was collected after a 4h fast from the retro-orbital sinus on the day of sacrifice. Livers were rapidly excised after perfusion with 10% phosphate buffered saline and either fixed in 10% formalin for histological examination, or snap-frozen in liquid nitrogen and stored at -70°C until further analysis. For tissue oxysterol and lipid peroxide analysis, approximately 200mg of liver was placed in antioxidant solution (10μM butylhydroxytoluene (BHT) and diethylene triamine pentaacetic acid in 95% ethanol) and frozen at -70°C until further analysis. This project was approved by the Animal Care and Use Committee of the University of Washington.

Analytical procedures: Plasma and liver cholesterol and triglycerides were measured using colorimetric kits. Plasma insulin was measured using an ELISA kit (Millipore, Billerica, MA). Alanine aminotransferase (ALT) was measured using an autoanalyzer through the Nutrition Obesity Research Center at the University of Washington; free fatty acid levels were measured colorimetrically (NEFA-C test kit, Wako, Richmond, VA). Liver lipid extraction was performed using a modified Folch technique (14). Hepatic thiobarbituric acid reactive substances (TBARS) concentration was measured in homogenates from 200mg of liver, as described previously (15).
Liver histology and immunohistochemistry: Formalin-fixed livers embedded in paraffin wax were sectioned and stained with hematoxylin and eosin (H & E) or Masson’s trichrome stains for histological analyses. Liver morphology was evaluated by a hepatopathologist (MMY) in a blinded manner. Macrophages were detected in liver sections immunohistochemically using a rat monoclonal antibody against Mac2 (titer 1:2500, Cedarlane Laboratories, Burlington, NC). Liver fibrosis was quantified in Trichrome stained sections of the liver. Area quantification for MAC2 and fibrosis were performed on digital images of immunostained liver sections using image analysis software (Image Pro Plus software, Media Cybernetics, Bethesda, MD). Liver cell apoptosis was assessed using the terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) assay according to the manufacturer’s instructions (ApopTag Peroxidase In Situ Apoptosis Detection Kit, Millipore, Billerica, MA). To determine the number of apoptotic hepatocytes, liver sections were quantified by counting the number of TUNEL-positive cells in 30 random microscopic fields (20X). Results are expressed as number of TUNEL-positive cells per field magnification.

Real-time quantitative PCR analysis: Total RNA was extracted from 100mg of liver tissue using TRI reagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer’s protocol. After spectroscopic quantification, 2µg of RNA was reverse-transcribed and cDNA thus obtained was analyzed by real-time quantitative PCR by standard protocols using the ABI 7900HT instrument in our laboratory. Primer and FAM probes for individual genes were purchased from Applied Biosystems (Assay-on-Demand, Life Technologies, Carlsbad, CA). Relative quantities of mRNA were calculated with GAPDH used as the reference gene and the amount of target gene was calculated using the ΔΔCt formula. Levels of the reference gene were not altered in all three animal groups.
Liquid chromatography electrospray ionization tandem mass spectrometry (LC/ESI/MS/MS) analysis of multiple species of lipid peroxidation products: Liver samples (~50 mg) were homogenized in 500µl of PBS with 100µM butylated hydroxytoluene and 1µM diethylene tetraamino pentaacetic acid (pH 7.0), followed by addition of an internal standard cocktail containing isotopically labeled 9(S)-HODE-d4, 13-(S)HODE-d4, 5-(S)HETE-d8, 12-(S)HETE-d8, 15-(S)HETE-d8, 20-(S)HETE-d6, arachidonic acid-d8 and linoleic acid-d4. The samples were incubated with an excess amount of sodium borohydride for 5 min, subjected to base hydrolysis with 1M KOH in methanol under nitrogen for 1h at 40 °C. The reaction was terminated by the addition of 2 ml of 10% acetic acid, lipids were extracted with chloroform/ethyl acetate (4:1, v/v) and the organic layers were pooled and dried.

Samples were subjected to reverse phase LC for HODEs, HETEs and arachidonic acid analysis utilizing Agilent 1200 LC system (Santa Clara, CA). Mass spectrometric experiments were performed using an Agilent technologies 6410 Triple Quadrupole system (Santa Clara, CA), equipped with an electrospray source. Quantification of oxidized fatty acids and their precursors were performed by comparing peak areas of the analyte of interest and their corresponding isotopically labeled internal standard. The levels of the oxidized fatty acid (HODEs and HETEs) were normalized to precursor fatty acids (linoleic and arachidonic acids) respectively. 8-iso-prostaglandin-F2α was measured by monitoring transition of the m/z 353 to 193 and m/z 357 to 193 to quantify native 8-iso-prostaglandin-F2α and its isotopically labeled internal standard 8-iso-prostaglandin-F2α-d4. Levels were normalized to arachidonic acid, the precursor fatty acid.

Determination of cholesterol oxidation products (oxysterols) by Gas Chromatography-Mass Spectrometry (GC/MS): Liver and diet samples (~200mg) were homogenized in a glass Tenbroeck homogenizer in 1ml PBS and 1ml aqueous 2% acetic acid (to inhibit lipolytic enzymes). The homogenate was extracted with chloroform/methanol (2:1) containing 100ppm
of BHT as an antioxidant. After centrifugation to separate the phases, the lower (chloroform) layer was removed and evaporated to dryness under nitrogen. Two parts methanol/1 part 6N KOH were added to the residue to saponify in the dark overnight. Sterols were extracted 3 times with diethyl ether and water. The extract was dried under nitrogen and derivatized with N,O-bis(trimethylsilyl) trifluoroacetamide in pyridine and heated at 60°C for 1hr. The sample was dried under nitrogen and taken up in hexane for analysis by GC/MS.

Statistical analyses: Data were analyzed using GraphPad Prism 5 program (GraphPad Software Inc, La Jolla, CA) and are presented as means and standard errors. Analysis of variance (ANOVA) with Bonferroni post hoc testing was used to detect differences among groups. Spearman’s correlative quotient was used to calculate associations. A p value <0.05 was considered as statistically significant.

RESULTS

Hepatic triglyceride accumulation increases with the addition of cholesterol to a high fat, high carbohydrate (diabetogenic) diet

We previously have shown that LDLR-/- mice fed a diabetogenic diet (DD) for 24 weeks gain weight, become hyperinsulinemic, and develop dyslipidemia manifested as high triglycerides and cholesterol (9). Addition of dietary cholesterol (DDC) worsened hyperinsulinemia without substantially worsening dyslipidemia. In the present study, we confirmed these findings (Table 1) and found equivalent weight gain in the DD and DDC groups. Liver weights were higher in both DD and DDC groups than in chow-fed animals (Table 1). Hypertriglyceridemia and hypercholesterolemia were not different between the obese LDLR-/- animals on the DD and DDC diets. Circulating free fatty acids were increased in both diabetogenic diet-fed groups, but were highest in the DDC group (p<0.001 vs chow, p<0.01 vs
DD, Table 1). Hepatic triglyceride content was increased in DD animals (Table 1, p<0.01 vs chow), but was higher in the DDC animals (p<0.001). Hepatic cholesterol levels were elevated only in the DDC group (Table 1). Circulating FFA levels correlated with hepatic triglyceride levels (r= 0.66, p=0.02) and plasma ALT levels (r=0.72, p=0.01) (Figure 1A, 1B). Thus, several metabolic alterations that have been implicated in the development of NAFLD were worsened by dietary cholesterol in obese LDLR-/- mice.

**Liver morphology is altered in LDLR-/- mice by the addition of cholesterol to a diabetogenic diet**

To determine the effect of dietary cholesterol on hepatic morphology, we performed histological examination of the livers. In general, the histopathological features required for a diagnosis of NASH in humans include macrovesicular steatosis (hepatocyte fat accumulation), lobular inflammation, fibrosis around hepatocytes and hepatic sinusoids and hepatocyte ballooning (16). Analysis of H&E stained liver sections from chow fed animals revealed normal liver histology without lipid accumulation in hepatocytes. In contrast, the DD group demonstrated diffuse macrovesicular steatosis in a non-zonal pattern (Figure 2A). Inflammation and fibrosis were seen only to a limited extent in the DD group. In the DDC animals, steatosis was both macrovesicular as well as microvesicular with a predominant centrilobular distribution. Moderate inflammatory cell foci (Figure 2B) and intrasinusoidal and pericellular fibrosis were seen to a greater extent in the DDC group (Figure 2C). Quantification of fibrosis in Trichrome stained sections revealed increased fibrotic areas in the DDC livers (chow 0.05%, DD 0.13% and DDC 0.21% of total area, p<0.05 chow vs DDC). Although neither group had hepatocyte ballooning, a feature seen in progressive human NASH, obese LDLR-/- mice showed several features suggestive of human NASH, which were heightened with dietary cholesterol.

**Dietary cholesterol alters hepatic inflammation in obese, insulin resistant LDLR-/- mice**
Hepatic inflammation is a key aspect of NASH, widely believed to be mediated through cytokines released from hepatocytes or resident macrophages otherwise known as Kupffer cells. Since macrophages have been implicated as a critical player in obesity-associated insulin resistance, we investigated the effects of dietary cholesterol on hepatic macrophages. Expression of the macrophage specific genes, F4/80, CD11b (not shown) and Mac2 were increased in the DDC group (Figure 3A). Expression levels of the pro-inflammatory cytokines TNF$\alpha$ and IL-6 mRNA also were increased in the DDC group (Figure 3B). Increased expression of MCP-1, a chemotactic factor for monocyte-macrophages and hepatic stellate cells was observed in both groups (Figure 3C). SAA1.1 and SAA2.1 are homologous isoforms of liver-derived proteins that are elevated in chronic inflammatory states (17), and have chemotactic activity (18). Expression of SAA1.1/SAA2.1 was increased in both groups but was significant in the DDC group only (Figure 3C). The trend was similar in the DD group but did not achieve statistical significance, likely because of the small number of animals per group.

Immunohistochemical analysis of liver sections for the pan-macrophage marker MAC2 showed increased staining in the DD group but was greater in the DDC group (Figure 3D and E). Taken together, these findings suggest that the addition of dietary cholesterol induces a greater degree of inflammation in the liver. Moreover, these findings parallel changes that we have shown previously in intra-abdominal (visceral) adipose tissue (9), suggesting that hepatic inflammation is an important component of diet-induced obesity in this mouse model.

Dietary cholesterol alters expression of hepatic metabolic genes in obese, insulin resistant LDLR--/- mice

To evaluate the effects of dietary cholesterol on hepatic triglyceride accumulation, we determined the hepatic expression of genes involved in fatty acid metabolism in the liver. No differences were detected in mRNA expression of FAS (Figure 4A) and ACC (not shown), two genes involved in fatty acid synthesis. Expression of SREBP1c, a transcription factor that
activates genes involved in lipogenesis, trended upwards in both obese groups of animals although it reached statistical significance only in the DDC group, again likely due to the small number of animals (Figure 4A). Similarly, expression of DGAT1, the enzyme which catalyzes the final step in triglyceride synthesis, was reduced in both groups of obese animals (Figure 4A). Expression of SREBP2, a transcription factor that regulates cholesterol biosynthesis, was unchanged across the three groups (Figure 4A). In contrast to several other studies (19, 20), we were not able to show an increase in liver CD36 mRNA expression (data not shown). Disturbed fatty acid beta-oxidation has been implicated in the pathogenesis of fatty liver (21). mRNA levels of CPT1α, a rate-limiting fatty acid transporter involved in beta-oxidation in the liver were increased in DD and DDC groups while PGC1α was increased in the DDC group only (Figure 4B) suggesting upregulation of the hepatic fatty acid oxidation machinery in this model.

Dietary cholesterol increases apoptosis in livers of obese LDLR-/- mice

Elevations in alanine aminotransferase (ALT) levels occur with NAFLD secondary to hepatocellular inflammation and injury. ALT levels were increased in the DD (Table 1, p<0.01 vs chow) but more so in the DDC group (p<0.001 vs chow and p<0.05 vs DD), suggesting a greater degree of hepatocyte injury with the addition of dietary cholesterol. ALT levels correlated strongly with hepatic triglyceride levels (r=0.85, p=0.0008). TUNEL staining revealed a few apoptotic cells in the livers of mice fed DD, which further increased in the DDC group of mice (Figure 5A). The number of apoptotic cells correlated with hepatic triglycerides (r=0.78, p=0.006, Fig 5B) and with ALT levels (r=0.69, p=0.02, Fig 5C).

Increased hepatic oxidative stress in obese mice fed added dietary cholesterol

Oxidative stress with increased production of reactive oxygen species has been observed in animal models and human NASH (22, 23). Lipids in the steatotic liver are targets of oxidation, and oxidative metabolites of the major fatty acids in vivo, arachidonic acid and linoleic
acid, are potent inflammatory mediators. Total hydroxyeicosatetraenoic acid (HETE) levels and hydroxyoctadecadienoic acid (HODE) levels account for the majority of stable oxidation products of arachidonic acid and linoleic acid respectively (24). In order to obtain a comprehensive analysis of lipid peroxidation, we analyzed structurally distinct oxidized fatty acid moieties including HODEs and HETEs and their precursor fatty acids from liver. Levels of 9-HODE and 13-HODE, products of linoleic acid oxidation, were significantly increased in the DDC group (Figure 6A). 13-HODE levels were highly correlated with hepatic triglyceride levels (r=0.90, p=0.002, Figure 6C). Modest elevations in several arachidonic acid oxidation products were observed in both obese groups. Levels of 8-iso-prostaglandin-F2α, derived from free radical mediated oxidation of arachidonic acid, trended higher in both DD and DDC groups than in chow fed animals, but the difference did not reach statistical significance (Figure 6D). Hepatic TBARS, a crude measure of lipid peroxidation products, also was increased in the DDC group (chow 1.39±0.13, DD2.90 ±1.74, DDC 5.98±3.6 nmol/mg protein, p<0.05 chow vs DDC, data not shown), confirming our findings with mass spectrometry.

Cholesterol oxidative products or oxysterols generated by auto-oxidation, enzymatic or nonenzymatic peroxidation of sterols also have been implicated in apoptosis and liver injury. Since dietary cholesterol can be modified by oxidation into oxysterols, we measured liver and dietary oxysterols such as 5,6,α-epoxy cholesterol, 5,6,β-epoxy cholesterol and 7-ketocholesterol. Levels of all these oxysterols were undetectable in the diet (data not shown). However, hepatic levels of α- and β-epoxy-cholesterol and 7-ketocholesterol were increased in the livers of mice on both diabetogenic diets, with α-epoxy-cholesterol being higher in the DDC group (Figure 6B). Taken together, these results reveal evidence of increased degree of oxidative stress in obese LDLR-/- mice which received the diabetogenic diet with added cholesterol.

DISCUSSION
We previously have shown that the addition of cholesterol to a diet rich in saturated fat and refined carbohydrate (diabetogenic diet) worsens adipose tissue inflammation, insulin resistance and systemic inflammation in LDLR-/- mice (9). Thus, this is a good model to study features of the metabolic syndrome. In the present study, we used the LDLR-/- mouse to evaluate the effect of these diets on the hepatic phenotype of the metabolic syndrome. Mice fed the diabetogenic diet without added cholesterol developed histological changes that closely resembled key features seen in human NAFLD, such as inflammation and fibrosis. Addition of dietary cholesterol resulted in worsened hepatic inflammation and fibrosis. Thus our data implicates dietary cholesterol as an important determinant of the severity of phenotypic changes that occur with progression of NAFLD.

Features of NAFLD in mouse models often do not fully mimic those seen in humans (4). Although the methionine-choline deficient diet commonly used to study NAFLD in mice results in hepatic fibrosis and inflammation, this model does not replicate human NASH effectively since mice on this diet lose weight and become more insulin sensitive (25), in contrast to the phenotype that occurs in the metabolic syndrome. Caloric excess by high fat and intragastric feeding appear to have many features of NASH, but technical challenges and failure to demonstrate reproducible results limit their widespread use (4). LDLR-/- mice develop obesity, dyslipidemia and insulin resistance similar to that seen in the metabolic syndrome in humans. In addition, we now show that inflammatory changes also occur in the liver, similar to what we have previously shown in adipose tissue (9). In line with a recent report (26), we found several histopathologic features reminiscent of human NAFLD in LDLR-/- mice fed a diabetogenic diet, with exacerbation by the addition of dietary cholesterol. Thus, LDLR-/- mice that develop obesity on a diet enriched in refined carbohydrate and saturated fat with added cholesterol have histological features that mimic NASH, together with a metabolic phenotype similar to that seen in obese humans. This model is therefore particularly attractive for studying the pathogenesis of liver disease in the metabolic syndrome, especially the role of dietary cholesterol.
A key feature in obesity associated with insulin resistance is adipose tissue inflammation, with accumulation of macrophages in fat depots in mice and humans (27, 28). We previously have shown that macrophages accumulate in the visceral adipose tissue of LDLR-/- mice fed these diabetogenic diets, and that the extent of macrophage accumulation and adipose tissue inflammatory gene expression were increased by cholesterol supplementation of the diabetogenic diet (9). Here we demonstrate that parallel changes occur in the livers of obese LDLR-/- mice, raising the possibility that similar mechanisms leading to inflammation and insulin resistance might be operative in both adipose tissue and liver. In this study, obese mice showed significant inflammatory changes in the liver, including increased expression of macrophage related genes and an increase in the number of MAC2 positive cells on immunostaining. While these effects were modest in mice which received the diabetogenic diet, they were amplified by addition of cholesterol to the diet. We also observed increased expression of MCP-1, a macrophage chemotactic factor; although expression levels were increased equally in both DD and DDC groups, suggesting that other chemotactic factors may play a role in liver monocyte-macrophage accumulation. Similarly, macrophage accumulation is not decreased in livers of MCP-1 deficient mice, although these mice are not obese, nor do they have fatty livers or insulin resistance (29).

Traditionally, the two hit hypothesis has been proposed for propagation of simple steatosis to NASH (30). Triglyceride accumulation in hepatocytes is widely accepted as an initial prerequisite for the development of NAFLD, which predisposes to cellular injury (31). Dietary cholesterol has been proposed as a second “hit” (8, 10). Other second insults that have been implicated in inflammatory liver damage and fibrogenesis seen in NASH include oxidative stress, mitochondrial dysfunction and upregulation of pro-inflammatory cytokines (5). In this study we have begun to address several of these potential contributors. Circulating FFA levels are increased in the metabolic syndrome and NAFLD in humans, and plasma levels correlate with disease severity (32). In our study, plasma FFAs correlated with hepatic triglyceride levels,
suggesting that circulating FFAs are an important source of hepatic triglycerides. Studies in rodents and humans have established fatty acid transporter CD36 as an important contributor to the pathogenesis of insulin resistance and NASH (19, 20). However, we were unable to confirm these findings in our study. One of the most consistent findings in the present study is that changes in the liver were exacerbated by the addition of cholesterol to the diet. Dietary cholesterol has been shown to induce hepatic inflammation in several studies. While other groups have evaluated the role of dietary cholesterol in NASH, several important differences exist between our studies and those of others. For example, short term feeding studies in mice using 2% dietary cholesterol showed that hepatic inflammation develops without steatosis (10). A hamster model of diet-induced obesity demonstrated steatosis and insulin resistance without significant weight gain (8). Sprague-Dawley rats fed a high fat diet with 2% cholesterol showed increased steatosis, inflammation and mitochondrial changes (33). Large doses of cholesterol in the diet prevent weight gain and are toxic to the liver, especially in conjunction with cholate (12). We used the LDLR-/- mouse model fed a diabetogenic diet without or with 0.15% cholesterol, an amount that appears to be well tolerated and did not blunt weight gain. Our use of lower non-toxic doses of cholesterol, has allowed us to gain additional insight mechanism that might be more relevant to the metabolic syndrome in humans. Genome based studies have shown that cholesterol feeding induces a wide range of alterations in hepatic metabolic and inflammatory genes, dependent upon the amount of cholesterol in the diet (34, 35).

The exact mechanisms by which dietary cholesterol induces hepatic inflammation and oxidative stress in our study are unclear and require further investigation. Cytotoxicity of free cholesterol is well established. A recent study showed that an important mechanism for cholesterol-mediated liver injury is the sensitization of hepatocyte mitochondria to cytokine mediated injury (36). Other potential mechanisms include endoplasmic reticular stress mediated apoptosis (37). Apoptotic cell death is a central feature of lipotoxic liver injury (38) and hepatocyte apoptosis correlates with disease severity in NASH (39). In our model, although
apoptosis occurred in livers of mice on both diabetogenic diets, dietary cholesterol increased apoptosis. Dietary cholesterol also increased circulating FFAs, which can trigger a pro-inflammatory response and induce lipoapoptosis (40).

Oxidative stress is a well-recognized mechanism contributing to disease progression in NAFLD (41-43). We detected a significant increase in certain subsets of lipid peroxidative products, such as 9-HODE, 13-HODE and 5-HETE, in mice fed dietary cholesterol. Generation of such lipid peroxides, either enzymatic or free radical mediated, are established markers of oxidative stress (44) and reflect the balance between pro-oxidant and antioxidant mechanisms within the tissue. As in our mouse model, increased levels of free-radical mediated linoleic acid oxidation products, namely 9- and 13-HODEs, have been detected in human NASH (44). In vitro evidence suggests that HODEs can stimulate apoptosis in monocytes (45). We also found increased 5-HETE levels and a statistically non-significant trend in 8-iso-prostaglandin-F2α derived from free radical lipid peroxidation. The strong correlation between hepatic 13-HODE and triglyceride levels provides evidence for a possible role for enzymatic pathways involving 15-lipoxygenase. Thus, these findings are consistent with the notion that both free radical as well as enzymatic oxidative pathways contribute to the formation of oxidized fatty acids in this model. In addition to these fatty acid by-products, cholesterol can also undergo auto-oxidation to oxysterols. Early studies indicated the substantial amounts of oxysterols can be present in the diet (46); however, oxysterols can also be formed endogenously by enzymatic conversion of cholesterol to a variety of cholesterol oxidation products (47). In our model we found increased levels of several oxysterols in livers from both groups of mice fed the diabetogenic diets, although we were unable to detect oxysterols in the diet. Oxysterols have been shown to be cytotoxic (48), and accumulation of oxysterols in the liver may have contributed to the increased hepatocyte apoptosis seen in our model. In vitro evidence suggests that certain oxysterols can trigger pro-inflammatory and pro-fibrotic changes in hepatocytes (49). Hypercholesterolemic apoE-/- mice have high hepatic oxysterol levels and increased susceptibility to liver injury and
fibrosis (49). Thus dietary cholesterol may have contributed to the fibrosis seen in these mice by conversion to oxysterols in vivo.

In conclusion, the LDLR-/- mouse has many characteristics seen in human NAFLD/NASH and therefore may serve as a model for studying the early and late changes occurring in the liver in obesity. We also show that dietary cholesterol exaggerates progression of simple steatosis to steatohepatitis by worsening hepatic inflammation. While the two-hit hypothesis finds wide acceptance in this process, in our model, dietary cholesterol appears to play a role in both triglyceride accumulation (first hit) and cytotoxicity, which may be a prelude to macrophage accumulation and hepatic inflammation, since changes in the liver mirror those seen in adipose tissue. Progression of simple steatosis to steatohepatitis might be the result of a complex interplay of multiple pathogenic factors such as hyperlipidemia, circulating FFAs, increased oxidized fatty acids and oxysterols, all of which contribute to inflammatory changes and cytotoxicity in the liver.
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Abbreviations: LDLR, LDL-receptor deficient, NAFLD, non-alcoholic fatty liver disease, NASH, non-alcoholic steatohepatitis, MCD, methionine-choline deficient, DD, diabetogenic diet, DDC, diabetogenic diet with cholesterol, TBARS, thiobarbituric acid reactive substance, TNFα, tumor necrosis factor-α, IL-6, interleukin-6, SAA1.1/2.1, FAS, fatty acid synthase, ACC, acetyl CoA carboxylase, SREBP-1c, sterol regulatory binding protein-1c, DGAT1, diacylglycerol:acyl transferase-1, SREBP2, sterol regulatory binding protein-2, CPT1α, carnitine palmitoyl transferase-1α, ACOX, acyl CoA oxidase, PGC1α, PPAR-γ coactivator 1α, HODE, hydroxyoctadecadienoic acid, HETE, hydroxyeicosatetraenoic acid, 7KC, 7-ketocholesterol.
FIGURE LEGENDS

Figure 1. Plasma free fatty acids play a key role in hepatic steatosis in obese mice. Free fatty acid levels (FFA) correlate strongly with, (A) hepatic triglyceride content and (B) plasma alanine aminotransferase (ALT), a marker of hepatocellular damage. Circles, DD; diamonds, DDC.

Figure 2. Dietary cholesterol worsens histological features of NASH in obese LDLR-/- mice. Representative images from chow, DD and DDC groups showing A, Steatosis (fat accumulation within hepatocytes) in H&E stained sections, inset 40X magnification and, B, Inflammatory foci in H&E sections, arrows indicate inflammatory cell clusters. Inset is 60X magnification. C, Fibrosis in Masson’s Trichrome stained sections for collagen which stains blue. DD, diabetogenic diet, DDC, diabetogenic diet with cholesterol.

Figure 3. Increased inflammation in livers of obese LDLR-/- mice is magnified by dietary cholesterol. (A) Macrophage gene expression, Emr1 and Mac2 mRNA; (B) Cytokine gene expression for Tnfa and Il6; (C) Chemotactic factor genes Mcp1 and Saa1.1/2.1, Open bars, chow diet (n=8), hatched bars, DD (n=10), and solid bars, DDC (n=10). (D) Representative immunostained photomicrographs of livers stained with macrophage-specific antibody MAC2 (red), 40x magnification, n=5-8 per group. Arrows point to areas of increased staining around portal triad (E) Quantification of MAC2 staining. Chow, closed squares, DD, closed circles, DDC diamonds. * p<0.05 vs chow, ** p<0.01 vs chow, $ p<0.05 vs DD.

Figure 4. Altered expression of hepatic metabolic genes in obese LDLR-/- fed dietary cholesterol. (A) Expression of genes involved in fatty acid synthesis, Fas, Srebp1c, Dgat1, Srebp2. (B) Genes involved in fatty acid oxidation Cpt1a, Acox1 and Pgc1a. Open bars, chow diet (n=8), hatched bars, DD (n=10), and solid bars, DDC (n=10). * p<0.05 vs chow, ** p<0.01 vs chow, *** p<0.001 vs chow.
Figure 5. Hepatocyte apoptosis in obese LDLR-/- fed dietary cholesterol. (A) TUNEL staining of liver sections, Open bars, chow diet (n=8), hatched bars, DD (n=10), and solid bars, DDC (n=10). * p<0.05 vs chow, ** p<0.01 vs DD, ***, p<0.001 vs chow. (B), The number of apoptotic cells correlate with hepatic triglyceride content and (C) with ALT levels, a marker of hepatocellular damage. Circles, DD, diamonds, DDC.

Figure 6. Evidence of increased oxidative stress in LDLR-/- mice is amplified by dietary cholesterol. (A) Lipid peroxidation as measured by mass spectrometry. Levels of HODEs and 5-HETE were increased in the DDC animals. (B) 8-isoprostaglandin-F2α levels in livers of lean and obese mice. (C) 13-HODE levels strongly correlate with hepatic triglyceride content. Circles represent DD, diamonds, DDC. (D) Cholesterol oxidative products (oxysterol) levels in liver were increased in obese mice. Open bars, chow diet (n=8), hatched bars, DD (n=10), and solid bars, DDC (n=10). * p<0.05 vs chow, ** p<0.01 vs chow, ***, p<0.001 vs chow, † p<0.01 vs DD. α epoxy, 5,6,α-epoxy cholesterol, β-epoxy, 5,6,β-epoxy cholesterol, 7 KC, 7 ketocholesterol.
Table 1. Metabolic variables in LDLR-/- mice after 24 weeks on the different diets

<table>
<thead>
<tr>
<th>Metabolic variable</th>
<th>Chow</th>
<th>DD</th>
<th>DDC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total body weight at end of study (g)</td>
<td>30.7±1.5</td>
<td>52.7±3.4***</td>
<td>52.2±1.6***</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>1.4±0.4</td>
<td>2.9±0.5**</td>
<td>3.2±0.4**</td>
</tr>
<tr>
<td>Epididymal fat pad weight (g)</td>
<td>0.2±0.1</td>
<td>1.6±0.2***</td>
<td>1.9±0.2***</td>
</tr>
<tr>
<td>Plasma insulin</td>
<td>0.7±0.2</td>
<td>3.9±1.3**</td>
<td>6.2±1.5**$</td>
</tr>
<tr>
<td>Cholesterol mg/dL</td>
<td>284±38</td>
<td>821±139**</td>
<td>875±47***</td>
</tr>
<tr>
<td>Triglycerides mg/dL</td>
<td>108±16</td>
<td>463±166**</td>
<td>511±139***</td>
</tr>
<tr>
<td>Free fatty acids nmol/L</td>
<td>0.15±0.1</td>
<td>0.8±0.3**</td>
<td>1.0±0.3**$</td>
</tr>
<tr>
<td>Hepatic triglycerides mg/g</td>
<td>58±14</td>
<td>72±38</td>
<td>135±62**$</td>
</tr>
<tr>
<td>Hepatic cholesterol mg/g</td>
<td>16.2±3</td>
<td>27±10</td>
<td>38±5*</td>
</tr>
<tr>
<td>ALT U/L</td>
<td>44±11</td>
<td>172±50**</td>
<td>244±106***$</td>
</tr>
</tbody>
</table>

Values represent means+/−SD. n=5-8 per group. DD, diabetogenic diet, DDC, diabetogenic diet with cholesterol. * p<0.05 vs chow, ** p<0.01 vs chow, ***p<0.001 vs chow,$ p<0.05 vs DD, # p<0.01 vs DD.
REFERENCES


hydroxyoctadecadienoic acid in monocytic cells. *Prostaglandins Leukot Essent Fatty Acids* 74: 283-293.


Figure 1

A

Hepatic Triglyceride content (mg/g tissue)

FFA mmol/L

r=0.66

p=0.02

B

ALT U/L

FFA mmol/L

r=0.72

p=0.01
Figure 2

A. STEATOSIS

B. INFLAMMATION

C. FIBROSIS
Macrophage genes

- Emr1 (F4/80)
- Mac2
- Tnf
- Il6
- Saa1.1/2.1
- Mcp1
- Emr1 (F4/80)

Cytokine genes

- Tnf
- Il6

Chemotactic factor genes

- Mcp1
- Saa1.1/2.1

Figure 3

D

Chow

DD

DDC

E

% MAC2 stained area

chow DD DDC

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Figure 4

A. Lipid synthesis genes

B. Fatty acid oxidation genes
Figure 5

A. Bar chart showing the number of TUNEL positive cells for Ch, DD, and DDC conditions. The bars are labeled with significance levels: * for p < 0.05, ** for p < 0.01, and *** for p < 0.001.

B. Scatter plot showing the correlation between hepatic triglyceride content (mg/g tissue) and TUNEL positive cells, with a Pearson's correlation coefficient (r) of 0.78 and p-value of 0.0006.

C. Scatter plot showing the correlation between ALT U/L and TUNEL positive cells, with a Pearson's correlation coefficient (r) of 0.69 and p-value of 0.02.
Figure 6

A. Oxidized fatty acid nM/ Precursor fatty acid mM

B. 8-iso-prostaglandin-F2α/ arachidonic acid µM

C. r=0.90 p=0.0002

D. Hepatic Triglyceride content (mg/g tissue)

- **p=0.07 by ANOVA
- *p=0.0002
- **
- ***