Published in final edited form as:

Gastroenterology. 2008 December ; 135(6): 2084–2095. doi:10.1053/j.gastro.2008.08.007.

Development of Non-alcoholic Steatohepatitis in Insulin Resistant L-SACC1 Mice

Sang Jun Lee^{1,§}, Garrett Heinrich^{1,§}, Larisa Fedorova², Qusai Y. Al-Share¹, Kelly J. Ledford¹, Mats A. Fernstrom¹, Marcia F. McInerney³, Sandra K. Erickson⁴, Cara Gatto-Weis⁵, and Sonia M. Najjar^{1,†}

¹ The Center for Diabetes and Endocrine Research and the Department of Physiology and Pharmacology at the College of Medicine at the University of Toledo, Health Science Campus, Toledo, Ohio, 43614 ² the Department of Medicine at the College of Medicine at the University of Toledo, Health Science Campus, Toledo, Ohio, 43614 ³ the Department of Pathology at the College of Medicine at the University of Toledo, Health Science Campus, Toledo, Ohio, 43614 ⁴ the Department of Medicinal and Biological Chemistry at the College of Pharmacy at the University of Toledo, Main Campus, Toledo, OH 43606 and ⁵ the Department of Medicine, University of California, and Veterans Affairs Medical Center, San Francisco, CA 94121

Abstract

Background & Aims—Liver-specific inactivation of CEACAM1 causes hyperinsulinemia and insulin resistance, which result from impaired insulin clearance, in L-SACC1 transgenic mice. These mice also develop steatosis. Because hepatic fat accumulation precedes hepatitis, lipid peroxidation and apoptosis in the pathogenesis of non-alcoholic steatohepatitis (NASH), we investigated whether a high-fat diet, by causing inflammation, is sufficient to induce hepatitis and other features of NASH in L-SACC1 mice.

Methods—L-SACC1 and wild-type mice were placed on high-fat diet for 3 months, then several biochemical and histological analyses were performed to investigate the NASH phenotype.

Results—A high-fat diet caused hepatic macrosteatosis and hepatitis, characterized by increased hepatic tumor necrosis factor (TNF α) levels and activation of the NF- κ B pathway, in L-SACC1 but not in wild-type mice. The high-fat diet also induced necrosis and apoptosis in the livers of the L-SACC1 mice.

Conclusions—A high-fat diet induced key features of human NASH in insulin-resistant L-SACC1 mice, validating this model as a tool to study the molecular mechanisms of NASH.

Introduction

About one third of adults in the United States are diagnosed with fatty liver disease, with 20–30% predicted to develop fibrosing steatohepatitis and 10% exhibiting the full spectrum of

[†]Address correspondence to: Sonia M. Najjar, Ph.D., College of Medicine, University of Toledo, Health Science Campus, 3000 Arlington Avenue, Mail stop 1008, Toledo, Ohio, 43614, Tel: (419) 383-4059, FAX: (419) 383-2871, e-mail: sonia.najjar@utoledo.edu.

[§]Authors have contributed equally to the work

Conflict of Interest: Authors declare that no conflicts of interest exist.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

nonalcoholic steatohepatitis (NASH). Incidence of the disease is expected to increase in parallel to increased prevalence of obesity.¹ With NASH progressing to cirrhosis and/or hepatocellular carcinoma and causing end-stage liver disease,² the disease is projected to become the leading liver disease and cause of liver transplantation due to cirrhosis in western countries.

NASH is characterized by hepatic macrosteatosis, inflammation and fibrosis. Its pathogenesis is not fully elucidated, but the most prevalent mechanism is the "two-hit" hypothesis.³ According to this hypothesis, hepatic steatosis initially develops (first hit) and predisposes to lipid peroxidation and inflammation, leading to hepatitis, apoptosis, fibrosis and ultimately, cirrhosis (second hit).

Activation of hepatic peroxisome proliferator-activated receptor α (PPAR α)-dependent mechanisms during fasting increases transcription of enzymes involved in fatty acid mitochondrial transport and β -oxidation, such as carnitine palmitoyl transferase1 (CPT1), to support gluconeogenesis. Some of these are co-regulated by PGC1 α (PPAR γ co-activator 1 α),⁴ which is mainly involved in promoting mitochondrial biogenesis and regulation of genes in the oxidative phosphorylation chain, such as the mitochondrial uncoupled protein-2 (UCP-2), which reduces ATP synthesis when activated by superoxides and the lipid peroxidation end products.⁵ Under conditions of obesity and prolonged high-fat intake, excessive fatty acid oxidation and lipid ω -peroxidation promote oxidative stress.⁶ Together with reduction of the mitochondrial glutathione (GSH) defense system against the cytotoxic effect of tumor necrosis factor α (TNF α), this activates IKK β -dependent NF- κ B inflammatory pathways and causes insulin resistance⁷, hepatitis,⁸ and mitochondrial dysfunction. It also predisposes to cell death and hepatocyte susceptibility to injury, and progressive liver diseases, such as NASH.⁹

Although NASH may develop in association with insulin resistance,^{10,11} the molecular relationship has not been clearly delineated,¹² in part due to the lack of an animal model that replicates adequately the human state. No animal model has developed NASH spontaneously, and few may develop some of the clinical manifestation of the disease.^{12,13} The methionine-choline deficient diet induces fibrosing steatohepatitis. However, humans with NASH do not exhibit methionine or choline deficiency and this diet does not cause insulin resistance. The relevance of the leptin-deficient *Ob/Ob* obese mouse in NASH pathogenesis has also been questionable because altered leptin signaling can itself modulate inflammatory response, fibrosis and hepatic lipid metabolism.¹⁴ Insight provided by the *Pten* mutant mouse is also limited because it is insulin sensitive and lean, and it develops massive steatosis by comparison to human NASH.¹⁵ The transgenic mouse with adipose tissue-specific expression of nuclear sterol regulatory element-binding protein 1c (SREBP-1c) displays marked steatosis with a liver histology similar to NASH.¹⁶ Because this mouse exhibits inherited lipodystrophy with hypoleptinemia and severe insulin resistance, it does not fully replicate the clinical manifestation of NASH. Thus, these experimental models failed to address adequately the role of insulin resistance in NASH pathogenesis.

In addition to insulin resistance, L-SACC1 mice with liver-specific overexpression of the dominant-negative S503A phosphorylation-defective mutant of the CarcinoEmbryonic Antigen-related Cell Adhesion Molecule 1 (CEACAM1) develop hepatic steatosis with increased hepatic triglyceride output and visceral obesity,¹⁷ resulting from impaired insulin clearance and hyperinsulinemia. This demonstrates that CEACAM1 promotes hepatic insulin clearance in a phosphorylation-dependent manner. CEACAM1 also acts as a co-inhibitory receptor after T cell activation to inhibit inflammation.¹⁸ Because this function depends on the phosphorylation of immunoreceptor tyrosine-based inhibition motifs within the cytoplasmic domain in addition to SHP1,¹⁹ we investigated whether L-SACC1 mice develop a NASH-like phenotype under conditions known to trigger inflammation, such as prolonged high-fat intake

(HF).²⁰ We herein report that L-SACC1 mice exhibit key features of obesity-related human NASH when fed HF for three months.

Materials and Methods

Animal Maintenance

Animals were kept in a 12-hour dark/light cycle and fed standard chow *ad libitum*. All procedures were approved by the Institutional Animal Care and Utilization Committee. Six month-old genetically matched wild type (WT) and L-SACC1 female mice were fed a standard chow (RD) or a HF diet (45% total calories from fat) for 3 months (Cat #D12451 Research Diets).

Metabolic Phenotype

Following an overnight fast, mice were anesthetized with sodium pentobarbital at 1100h. Whole venous blood was drawn from the retro-orbital sinuses to measure fasting glucose levels using a glucometer (Accu-chek; Roche Applied Science), serum insulin and leptin levels by radioimmunoassays (Linco Research), serum FFAs (NEFA C kit-Wako), triglycerides (Infinity Triglycerides-Sigma) and cholesterol (Pointe Scientific), and free cholesterol (Wako). Serum alanine- and aspartate aminotransferases levels (ALT and AST, respectively) were measured per manufacturer's instructions (Biotron Diagnostic). Visceral adipose tissue was excised, weighed, and visceral adiposity was expressed as percentage of total body weight. Hepatic total cholesterol and free cholesterol were measured using Infinity cholesterol reagent (Thermo Electron) and free cholesterol reagent (Wako), respectively.²¹

Liver Histology

Histological examination was established using hematoxylin-eosin (H&E) of formalin-fixed paraffin-embedded liver. The degree of steatosis and lobular inflammation was graded on a 0–3 scale, with 3 being the highest, according to the NASH scoring system, which was recently proposed by the NIDDK-NASH Clinical Research Network.²²

Liver Staining

Deparaffinized and rehydrated slides were incubated in 0.1% solution of Sirius Red (Sigma, Direct Red 80) in saturated picric acid in the dark for 1 hour and mounted with resin. For the TUNEL assay, sections were stained with ApopTag Plus Peroxidase Apoptosis Detection Kit (Chemicon International) per manufacturer instructions. For immunohistochemical analysis, liver sections were blocked in 1.5% horse serum (Vector Laboratories) for 30 minutes and probed with FITC-conjugated anti-mouse antibodies to F4/80 (1:20, eBioscience), and CD3 or CD4 (1:100, BD Pharmingen) for 1 hour in the dark. Nuclei were counterstained with propidium iodide. Eight-to-ten random fields per section were counted. Images were captured using a Leica TCS SP5 broadband confocal microscope.

GSH Assay

Reduced glutathione was measured using the Bioxytech GSH-400 kit (OXISResearch).²³ Briefly, liver tissue was homogenized in 5% metaphosphoric acid prior to adding a chromogenic reagent (4-chloro-1-methyl-7-trifluoromethyl-quinolinium methylsulfate) and 30% NaOH, followed by incubation at room temperature for 10 minutes in the dark and measurement at A400nm.

Nitrite Assay

Serum nitrite levels were measured using the Griess reagent system (Promega). Briefly, equal amounts of sulfanilamide solution and N-1-naphthylethylenediamine dihydrochloride solution

were added to equal volumes of serum, and allowed to incubate at room temperature for 10 minutes before measurement at A540nm.

Thiobarbituric Acid Reactive Substances (TBARS) Assay

Lipid peroxidation was measured as described¹³ with modifications. Briefly, liver tissue was homogenized in 1.15% KCl before centrifugation at 10,000 rpm at 4°C for 10 minutes. The supernatant (100μl) was added to 8.1% SDS, 20% acetic acid, and 0.8% thiobarbituric acid, followed by heating at 95°C for 60 minutes. n-butanol-pyridine (15:1) was added to the cool mix prior to centrifugation at 4000 × g for 10 minutes and the upper layer was measured at A532nm.

Western Analysis

Total tissue lysates or 10μg serum lysates (for ApoB) were analyzed by 4–12% gradient SDS-PAGE (Invitrogen) prior to Western analysis with polyclonal antibodies against PPARα, PGC-1α, UCP-2 (Santa Cruz Biotechnology), CYP2E1 (Chemicon International), ApoB48/100 (Chemicon International), fatty acid synthase (FAS),²⁴ NPC-1 (Abcam), procollagen (SouthernBiotech) and p65 NF-κB phosphoserine (Ser 536), p65 NF-κB and PKCζ phosphothreonine-Thr 410/403 (Cell Signaling Technology), in addition to monoclonal antibodies against PPARγ, PKCζ GAPDH (Santa Cruz) and Actin (Sigma). Membranes were incubated with horseradish peroxidase-conjugated anti-IgG antibody and proteins detected by enhanced chemiluminescence (Amersham Pharmacia Biotech) and quantified by densitometry.

Northern Analysis

Liver mRNA was extracted with Trizol (Invitrogen) and the MicroPoly(A) Pure kit (Ambion) before probing with Random Primed-labelled cDNAs (Roche) for CPT1, phosphoenolpyruvate carboxykinase (PEPCK), pyruvate dehydrogenate kinase (PDK-4), glucose-6-phosphatase (G-6-Pase), sterol regulatory element-binding protein 1c (SREBP1c), and tumor necrosis factor α (TNFα) before reprobing with GAPDH cDNA to normalize for the amount loaded.

Real Time PCR

RNA was extracted using RNeasy Mini Kits (Qiagen) before TNFα cDNA was synthesized by M-MLV reverse transcriptase, and its level quantitated by Absolute SYBR Green real-time PCR mastermix (Thermo Fisher Scientific) in a Bio-Rad I-cycler. TNFα level was normalized with GAPDH using the following primers:

TNFα forward: TTCTGTCTACTGAACTTCGGGGTGATCGGTCC

TNFα reverse: GTATGAGATAGCAAATCGGCTGACGGTGTGGG

GAPDH forward: CCAGGTTGTCTCCTGCGACT

GAPDH reverse: ATACCAGGAAATGAGCTTGACAAAGT

Statistical Analysis

Data were analyzed with Statview software (Abacus) using one-factor analysis of variance analysis. *P*<0.05 was considered to be statistically significant.

Results

High-Fat Diet Exacerbates the Metabolic Derangement in L-SACC1 mice

As previously reported,^{17,25} nine month-old female L-SACC1 mice develop higher body weight and visceral obesity by comparison to their WT counterparts (Table 1). Prolonged HF intake for 3 months increases visceral fat and consequently, serum leptin levels, in both groups of mice, with a stronger effect on WT mice (Table 1). Moreover, HF increases serum insulin and free fatty acids (FFA) levels by ~2-fold in WT, but not L-SACC1 mice (Table 1).

Consistent with insulin resistance,^{17,25} fed glucose level is higher in RD-fed L-SACC1 than WT mice (Table 1). Elevation in hepatic *Pepck* and *G-6-Pase* mRNA levels at fasting^{17,25}, but not post-prandial state in mice fed a chow-diet (RD) (Figure 1) suggests that L-SACC1 mice are geared toward gluconeogenesis, and hence, are predisposed to develop fasting hyperglycemia in response to HF diet. In fact, HF causes a ~2-fold increase (Table 1) in fasting glucose level of L-SACC1 mice together with a significant increase in hepatic post-prandial mRNA levels of gluconeogenic enzymes: *Pepck*, *G-6-Pase* and *Pdk-4* (Figure 1). In contrast, HF does not alter fasting blood glucose level in WT mice. Instead, it causes a more marked increase in fed glucose level (by ~2-fold) than in L-SACC1 mice (Table 1).

High-fat Diet Alters Lipid Metabolism in L-SACC1 Mice

As previously reported,¹⁷ hepatic triglyceride content is higher in L-SACC1 than WT mice (Table 1), but in response to HF, it is increased to the same extent (by ~1.8-fold) in both mouse groups (Table 1). Because hyperinsulinemia increases the transcription of lipogenic enzymes,²⁶ this could result, at least in part, from increased de novo lipogenesis, as suggested by the ~2 to 3-fold increase in hepatic mRNA levels of *Srebp-1c* (Figure 1C) and the protein level of one of its target, FAS (Figure 2A). Basal protein expression of PPAR γ , a key regulator of lipogenesis in adipocytes, is higher in L-SACC1 relative to WT livers (by ~3-fold) (Figure 2A; RD-fed L-SACC1 versus WT) and does not increase further by HF (Figure 2A; HF- versus RD-fed L-SACC1 mice). The increase in hepatic de novo lipogenesis in L-SACC1 mice correlates with higher serum triglyceride levels when mice are fed RD (Table 1), but not HF, which causes a marked decrease in serum triglyceride (Table 1) and ApoB100/ApoB48 protein levels (Figure 2B). The latter suggests reduced hepatic output of triglycerides by HF in L-SACC1 mice. In WT mice, HF does not increase serum triglyceride levels despite increasing hepatic production, as indicated by elevated *Srebp-1c* (Figure 1), FAS and PPAR γ levels (Figure 2A). With serum ApoB100/Apo48 protein content being normal (Figure 2B), reduction in serum triglyceride could result from substrate redistribution from liver to white adipose tissue, as suggested by increased visceral obesity and elevated release of FFA and leptin in HF-fed WT mice (Table 1).

Whereas total body and intestinal *In vivo* cholesterol synthesis are unchanged, hepatic cholesterol synthesis is higher (by ~2-fold) in chow-fed L-SACC1 mice (Table 1). This leads to an increase in hepatic content of cholesterol esters with a reciprocal decrease in free cholesterol levels, resulting in a net balance of total cholesterol levels in liver and serum from L-SACC1 mice (Table 1). Whereas HF does not change hepatic cholesterol levels, it reduces significantly (by ~50%) hepatic protein content of NPC1, a late endosomal cholesterol traffic protein, in L-SACC1, but not WT mice (Figure 2A).

H&E staining of liver sections reveals that HF differentially modifies the histological manifestation of steatosis in L-SACC1 and WT mice (Figure 2C). HF markedly increases hepatic steatosis in L-SACC1 mice (score of 1.33 $\pm$ 0.28 versus 0.30 $\pm$ 0.12 in RD-fed; $P < 0.05$), and changes its histology from microsteatosis (Figure 2C, block 3) to macrosteatosis (Figure

2C, block 4). In WT mice, however, HF increases steatosis (0.58 ± 0.30 versus 0.00 ± 0.00 in RD-fed; $P < 0.05$), while preserving its microvesicular histology (Figure 2C, block 2).

High-Fat Diet Induces Hepatitis in L-SACC1 Mice

H&E staining of liver sections reveals that HF causes scattered inflammatory infiltration in L-SACC1 (Figure 3A, block 4 versus 3; score of 1.25 ± 0.28 versus 0.40 ± 0.19 in RD-fed; $P < 0.05$), with altered hepatocellular architecture. In contrast, WT mice exhibit few, if any inflammatory islands (Figure 3A, block 2 versus 1), with no significant score difference (0.42 ± 0.15 versus 0.50 ± 0.00 in RD-fed; $P > 0.05$), and maintain normal cell architecture in response to HF diet. Consistently, HF increases hepatic *Tnfa* mRNA levels (by ~2-fold) in L-SACC1, but not WT mice, as assessed by Northern analysis (Figure 3B). Quantitative RT-PCR reveals that HF diet also increases *Tnfa* mRNA levels in white adipose tissue derived from L-SACC1 (8.86 ± 1.42 versus $3.83 \pm 0.99\%$ *Gapdh* in RD-fed; $P = 0.023$), but not WT mice (1.54 ± 0.03 versus $1.99 \pm 0.05\%$ *Gapdh* in RD-fed; $P > 0.05$).

Consistent with a role for TNF α in activating IKK- β , a redox-sensitive kinase that upregulates pro-inflammatory pathways,²⁷ HF markedly activates NF- κ B in L-SACC1 liver, as indicated by a ~4-fold increase in the phosphorylation of NF- κ B (Figure 3C). In contrast, HF does not activate NF- κ B in WT mice to a significant extent (Figure 3C). In support of PKC- ζ playing an important role in NF- κ B activation,²⁸ HF increases phosphorylation (activation) of PKC- ζ in L-SACC1, but not WT liver (Figure 3C).

In further support of increased release of TNF α from macrophages, immunostaining analysis with F4/80 reveals a larger macrophage pool in the liver of HF-fed L-SACC1 mice (Figure 4A, block 4 versus 1–3, green stain). Additionally, HF induces CD3⁺/CD4⁺T cell population in L-SACC1, but not WT mice, as assessed by immunohistochemical analysis (Figure 4B). Taken together, the data indicate that HF diet induces hepatitis in L-SACC1 mice, and that this may implicate increase in TNF α and CD4⁺Tcell population, both of which are critical players in NASH pathogenesis.

High-Fat Diet Causes Oxidative Changes in L-SACC1 Mice

Western analysis reveals that hepatic PPAR α , PGC1 α and UCP2 protein levels are higher in L-SACC1 than WT mice by ~2-fold (Figure 5A). Consistent with increased activation of PPAR α by HF,²⁹ prolonged fat feeding induces PPAR α hepatic protein levels by ~2-fold in both mouse groups (Figure 5A, HF versus RD lanes), and induces *Cpt1* mRNA levels with a more statistically significant effect in L-SACC1 mice (Figure 1A). This suggests increased fatty acid oxidation in HF-fed L-SACC1 mice.

The protein level of hepatic CYP2E1, a member of the microsomal cytochrome p450 that is involved in the metabolism of long chain fatty acids (lipooxygenation) and microsomal lipid ω -peroxidation, is comparable in RD-fed L-SACC1 and WT mice (Figure 5A). HF increases CYP2E1 protein level by ~3-fold in L-SACC1, but not WT mice (Figure 5A). Moreover, lipid peroxidation, as assessed by TBARS concentration in liver lysates, is highly elevated in HF-fed as compared to RD-fed L-SACC1 and WT mice (Figure 5B). This suggests initiation of oxidative changes in L-SACC1 liver by HF diet.³⁰ In support of this hypothesis, the level of serum nitrite, a nitric oxide (NO) oxidation product, is decreased in L-SACC1, as compared to WT mice (Figure 5B), suggesting lower NO bioavailability in these mice, as in other models of obesity.³¹ Consistent with the positive effect of TNF α on nitrite production,³² HF induces a more marked increase in serum nitrite level in L-SACC1 than WT mice (Figure 5B; HF versus RD) and decreases hepatic GSH levels in L-SACC1, but not WT mice (Figure 5B). Thus, HF promotes a nitroso-redox imbalance in L-SACC1 mice.

High-Fat Diet Induces Necrosis and Apoptosis in L-SACC1 Mice

Western analysis of liver lysates indicates that poly(ADP-ribose) polymerase (PARP) is cleaved into its smaller 85kDa subunit only in HF-fed L-SACC1 mice, but not in the other groups (Figure 6A). This suggests that early apoptosis develops in L-SACC1 mice only after HF feeding.³³ This observation is supported by TUNEL staining, which detects histological changes only in HF-fed L-SACC1 mice (Figure 6B, block 4-arrowheads versus block 1–3). H&E staining reveals that livers from HF-fed L-SACC1 mice, but not others, also exhibit necrosis (Figure 6B, block 5-arrowheads and Figure 3, block 4).

Development of Early Fibrosis in L-SACC1 Mice

Liver lysates from L-SACC1 mice exhibit elevated basal procollagen protein content (Figure 7A, RD-fed L-SACC1 versus WT), with a NASH-like "chicken-wire" pattern of the Sirius red stain (Figure 7B, block 3). In addition to inducing procollagen content (Figure 7A), HF increases chicken-wire fibrogenic changes in L-SACC1, but not WT mice (Figure 7B, block 4 versus 3).

Discussion

The mechanisms underlying NASH and the role of insulin resistance in its pathogenesis are poorly understood, in part due to the lack of an experimental model replicating the full spectrum of the disease.¹² We show that L-SACC1 mice, which exhibit insulin resistance, visceral obesity and hepatic steatosis,¹⁷ also develop early hepatic fibrosis together with a mild increase in serum ALT and AST (Table 1), and hepatic TNF α levels. When fed HF, they develop other key features of NASH, including macrosteatosis followed by hepatitis, lipid peroxidation, oxidative stress and hepatocellular injury.

As in human NASH,¹¹ HF induces hepatic fatty acid oxidation and gluconeogenesis in L-SACC1 mice and causes fasting hyperglycemia. The increase in fatty acid oxidation fails to decrease hepatic triglyceride content, owing to increased triglyceride production and decreased secretion, as indicated by elevation of *Srebp-1c* mRNA and reduction of ApoB100 levels,³⁴ respectively.

CEACAM1 mutation increases hepatic cholesterol synthesis and esterification in the endoplasmic reticulum, which could be facilitated by increased supply of de novo synthesized fatty acids in the absence of CEACAM1.²⁴ It is possible that increase in esterification occurs to prevent increase in free cholesterol level and protect against its cytotoxic effect.^{35,36} This assigns a role to CEACAM1 in integrating cholesterol metabolism and fatty acid synthetic pathways.

It is unlikely that increase in cholesterol ester plays a role in the progression to steatohepatitis since this appears to require accumulation of free cholesterol in mitochondria.³⁷ We did not measure mitochondrial free cholesterol content, but reduced NPC1 levels may partition it from cytosolic lipid droplets to mitochondria³⁸ in HF-fed L-SACC1 mice. As suggested by null mutation of *NPC1*,³⁷ this could deplete mitochondrial GSH stores and increase sensitivity to the cytotoxic effect of the pro-inflammatory cytokine, TNF α , the level of which is elevated in these mice.

Elevation in hepatic *Tnfa* mRNA could result from increased pool of resident macrophages in response to increased FFA supply³⁹ and excessive lipid accumulation in the hepatocyte, which could induce local inflammatory response in HF-fed L-SACC1 mice.⁴⁰ It is possible that the intrahepatic inflammatory milieu is modulated by the release of adipokines, as suggested by increased *Tnfa* mRNA in visceral white adipose tissue of L-SACC1, but not WT mice.

In agreement with experimental animal models^{13,41} and humans^{42,43} with metabolic derangement, lipid peroxidation is also increased in HF-fed L-SACC1. Together with a more marked increase in serum nitrite levels, this suggests that HF causes nitroso-redox imbalance and oxidative stress that may pave the way for peroxidative events associated with necrotic damage⁴⁴ and apoptosis⁴⁵ in L-SACC1 mice. We propose that HF causes these pathologies in L-SACC1, not WT mice, by inducing hepatic TNF α ⁴⁶ and increasing sensitivity to its cytotoxic effect through alterations of the GSH-based mitochondrial defense system. Consistent with a role for TNF α -dependent activation of IKK- β in oxidative stress and inflammation of NASH,³⁹ HF activates NF- κ B pathways to a larger extent in L-SACC1 than WT mice.

Steatohepatitis involves a Th1 cytokine response, characterized by increased cytokine release from intrahepatic CD4⁺T cells.^{20,47,48} Although the activation state of these cells was not examined, we observed an increase in their population in L-SACC1 mice, as has been reported for *Ob/Ob* mice.⁴⁰ Because CEACAM1 has been implicated in the anti-inflammatory response to T cell activation,¹⁸ it is possible that inactivating CEACAM1 in hepatocytes limits the CEACAM1-dependent inhibitory responses in T lymphocytes and leads to a robust inflammatory response to cytokines in L-SACC1 mice.

Chow-fed L-SACC1 mice exhibit insulin resistance and hepatic steatosis, due to accumulation of triglycerides, which results from increased synthesis and reduced secretion. Based on the normal physiology of insulin action, one would predict that insulin resistance would not be associated with increased hepatic triglyceride content. This prediction is borne out in the LIRKO mouse.⁴⁹ However, unlike LIRKO, L-SACC1 mice have reduced, but not absent insulin receptor signaling, which may lead to the peculiar admixture of insulin sensitivity (in the context of increased lipogenesis) and resistance (in terms of increased fatty acid oxidation and altered glucose homeostasis) which is characteristic of NASH.

In support of the hypothesis that insulin resistance is an independent predictor for fibrosis in NASH,⁵⁰ chow-fed L-SACC1 mice develop NASH-like fibrogenic changes that can be attributed to elevation in both TNF α and leptin.⁴⁶ In fact, increased leptin levels do not play a direct role in the pathogenesis of NASH,⁵¹ but can exacerbate the effects of TNF α .⁵² Additionally, L-SACC1 mice exhibit basal mild elevation in AST and ALT levels. Consistent with observations in a rabbit model of steatohepatitis,¹³ high-fat intake does not further elevate AST and ALT levels. With the majority of NASH patients exhibiting normal AST levels,⁵³ this suggests that altered AST levels in L-SACC1 mice may closely parallel the mildly elevated levels in basal TNF α , leptin and fibrogenic changes, which in turn, are likely to be associated with visceral obesity, insulin resistance and hepatic steatosis. We propose that these cellular, metabolic and fibrogenic changes “precondition” the mice to develop the overt NASH phenotype in parallel to increased inflammatory activation and oxidative stress when cued by elevation in the cytotoxic effect of TNF α in response to the HF diet.

The insulin resistant L-SACC1 mice are predisposed to developing NASH in response to HF. This could be due to altered lipid metabolism, which leads to reduced hepatic GSH-based defense against TNF α , and to increased inflammatory response to TNF α in the absence of the anti-inflammatory effect of CEACAM1. By demonstrating a dual role for CEACAM1 in inhibiting inflammatory response and reducing steatosis in liver, our observations indicate that a reduction in CEACAM1 may serve as a molecular link between insulin resistance and NASH.

Acknowledgments

We thank Dr. Sandrine Pierre for advice on lipid peroxidation assay, Dr. R. Mark Wooten for his guidance in inflammation analysis, and Dr. Andrea Kalinoski for help in confocal microscopy. We also thank Anthony DeAngelis, Jill M. Schroeder-Gloeckler, Sadeesh Ramakrishnan, Jehnan Liu and Jennifer Kalisz (Najjar laboratory) and Steve Lear (Erickson laboratory) for excellent technical assistance.

This work was supported by grants from the National Institutes of Health (DK 54254 to SM Najjar and DK072187 to SK Erickson), the American Diabetes Association (to SM Najjar), the United States Department of Agriculture (USDA 38903-02315) (to SM Najjar and MF McInerney), and by a Merit Award from the Department of Veteran's Affairs (to SK Erickson).

Nonstandard abbreviations used

NASH	nonalcoholic steatohepatitis
WT	wild type mouse
L-SACC1	liver-specific S503A CEACAM1 mutant mouse
RD	regular diet
HF	high-fat diet
TG	triglyceride
FFA	free fatty acids
FAS	fatty acid synthase
PPAR α	γ , peroxisome proliferator-activated receptor α , γ
PGC1α	PPAR γ co-activator 1 α
UCP2	uncoupling protein 2
CPT-1	carnitine palmitoyl transferase 1
G-6-Pase	glucose-6-phosphatase
PDK-4	pyruvate dehydrogenate kinase
PEPCK	phosphoenolpyruvate carboxykinase
SREBP1c	sterol regulatory element-binding protein 1c
NPC-1	Niemann Pick type C1
ALT	alanine aminotransferase
AST	aspartate aminotransferase
GSH	glutathione
CYP2E1	cytochrome p450 enzyme
TNFα	tumor necrosis factor- α
PARP	poly(ADP-ribose) polymerase
H&E staining	hematoxylin-eosin staining, pten, phosphatase and tensin homolog on chromosome 10

References

1. Farrell GC, Larter CZ. Nonalcoholic fatty liver disease: from steatosis to cirrhosis. *Hepatology* 2006;43:S99–S112. [PubMed: 16447287]
2. Hui JM, Kench JG, Chitturi S, et al. Long-term outcomes of cirrhosis in nonalcoholic steatohepatitis compared with hepatitis C. *Hepatology* 2003;38:420–427. [PubMed: 12883486]

3. Day CP, James OF. Steatohepatitis: a tale of two "hits"? *Gastroenterology* 1998;114:842–845. [PubMed: 9547102]
4. Yoon JC, Puigserver P, Chen G, et al. Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1. *Nature* 2001;413:131–8. [PubMed: 11557972]
5. Echtaay KS, Murphy MP, Smith RA, et al. Superoxide activates mitochondrial uncoupling protein 2 from the matrix side. Studies using targeted antioxidants. *J Biol Chem* 2002;277:47129–47135. [PubMed: 12372827]
6. Chavin KD, Yang S, Lin HZ, Chatham J, et al. Obesity induces expression of uncoupling protein-2 in hepatocytes and promotes liver ATP depletion. *J Biol Chem* 1999;274:5692–5700. [PubMed: 10026188]
7. Arkan MC, Hevener AL, Greten FR, et al. IKK-beta links inflammation to obesity-induced insulin resistance. *Nat Med* 2005;11:191–198. [PubMed: 15685170]
8. Dela Pena A, Leclercq I, Field J, et al. NF-kappaB activation, rather than TNF, mediates hepatic inflammation in a murine dietary model of steatohepatitis. *Gastroenterology* 2005;129:1663–1674. [PubMed: 16285964]
9. McClain CJ, Mokshagundam SP, Barve SS, et al. Mechanisms of non-alcoholic steatohepatitis. *Alcohol* 2004;34:67–79. [PubMed: 15670668]
10. Marchesini G, Marzocchi R. Metabolic Syndrome and NASH. *Clin Liver Dis* 2007;11:105–117. [PubMed: 17544974]
11. Abdelmalek MF, Diehl AM. Nonalcoholic fatty liver disease as a complication of insulin resistance. *Med Clin North Am* 2007;91:1125–1149. ix. [PubMed: 17964913]
12. Green RM. NASH Hepatic metabolism and not simply the metabolic syndrome. *Hepatology* 2003;38:14–17. [PubMed: 12829980]
13. Otagawa K, Kinoshita K, Fujii H, et al. Erythrophagocytosis by liver macrophages (Kupffer cells) promotes oxidative stress, inflammation, and fibrosis in a rabbit model of steatohepatitis: implications for the pathogenesis of human nonalcoholic steatohepatitis. *Am J Pathol* 2007;170:967–980. [PubMed: 17322381]
14. Leclercq IA, Farrell GC, Schriemer R, et al. Leptin is essential for the hepatic fibrogenic response to chronic liver injury. *J Hepatol* 2002;37:206–13. [PubMed: 12127425]
15. Kurlawalla-Martinez C, Stiles B, Wang Y, et al. Insulin hypersensitivity and resistance to streptozotocin-induced diabetes in mice lacking PTEN in adipose tissue. *Mol Cell Biol* 2005;25:2498–2510. [PubMed: 15743841]
16. Nakayama H, Otabe S, Ueno T, et al. Transgenic mice expressing nuclear sterol regulatory element-binding protein 1c in adipose tissue exhibit liver histology similar to nonalcoholic steatohepatitis. *Metabolism* 2007;56:470–475. [PubMed: 17379003]
17. Poy MN, Yang Y, Rezaei K, et al. CEACAM1 regulates insulin clearance in liver. *Nature Genet* 2002;30:270–276. [PubMed: 11850617]
18. Gray-Owen SD, Blumberg RS. CEACAM1: contact-dependent control of immunity. *Nat Rev Immunol* 2006;6:433–446. [PubMed: 16724098]
19. Nagaishi T, Pao L, Lin SH, Iijima H, et al. SHP1 Phosphatase-Dependent T Cell Inhibition by CEACAM1 Adhesion Molecule Isoforms. *Immunity*. 2006
20. Li Z, Soloski MJ, Diehl AM. Dietary factors alter hepatic innate immune system in mice with nonalcoholic fatty liver disease. *Hepatology* 2005;42:880–4885. [PubMed: 16175608]
21. Erickson SK, Lear SR, Dean S, et al. Hypercholesterolemia and changes in lipid and bile acid metabolism in male and female cyp7A1-deficient mice. *J Lipid Res* 2003;44:1001–1009. [PubMed: 12588950]
22. Bondini S, Kleiner DE, Goodman ZD, et al. Pathologic Assessment of Non-alcoholic Fatty Liver Disease. *Clin Liver Dis* 2007;11:17–23. [PubMed: 17544969]
23. Kellogg AP, Wiggin TD, Larkin DD, et al. Protective effects of cyclooxygenase-2 gene inactivation against peripheral nerve dysfunction and intraepidermal nerve fiber loss in experimental diabetes. *Diabetes* 2007;56:2997–3005. [PubMed: 17720896]
24. Najjar SM, Yang Y, Fernstrom MA, et al. Insulin acutely decreases hepatic fatty acid synthase activity. *Cell Metab* 2005;2:43–53. [PubMed: 16054098]

25. Dai T, Abou-Rjaily GA, Al-Share' QY, et al. Interaction between altered insulin and lipid metabolism in CEACAM1-inactive transgenic mice. *J Biol Chem* 2004;279:45155–45161. [PubMed: 15316023]
26. Horton JD, Goldstein JL, Brown MS. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J Clin Invest* 2002;109:1125–1131. [PubMed: 11994399]
27. Cai D, Yuan M, Frantz DF, et al. Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. *Nat Med* 2005;11:183–190. [PubMed: 15685173]
28. Manicassamy S, Gupta S, Huang Z, et al. Protein kinase C-theta-mediated signals enhance CD4+T cell survival by up-regulating Bcl-xL. *J Immunol* 2006;176:6709–6716. [PubMed: 16709830]
29. Patsouris D, Reddy JK, Muller M, et al. Peroxisome proliferator-activated receptor alpha mediates the effects of high-fat diet on hepatic gene expression. *Endocrinology* 2006;147:1508–1516. [PubMed: 16357043]
30. Robertson G, Leclercq I, Farrell GC. Nonalcoholic steatosis and steatohepatitis. II Cytochrome P-450 enzymes and oxidative stress. *Am J Physiol Gastrointest Liver Physiol* 2001;281:G1135–1139. [PubMed: 11668021]
31. Saraiva RM, Minhas KM, Zheng M, et al. Reduced neuronal nitric oxide synthase expression contributes to cardiac oxidative stress and nitroso-redox imbalance in ob/ob mice. *Nitric Oxide* 2007;16:331–338. [PubMed: 17307368]
32. Alloati G, Penna C, De Martino A, et al. Role of nitric oxide and platelet-activating factor in cardiac alterations induced by tumor necrosis factor-alpha in the guinea-pig papillary muscle. *Cardiovasc Res* 1999;41:611–619. [PubMed: 10435033]
33. Simbulan-Rosenthal CM, Rosenthal DS, Iyer S, et al. Transient poly(ADP-ribosyl)ation of nuclear proteins and role of poly(ADP-ribose) polymerase in the early stages of apoptosis. *J Biol Chem* 1998;273:13703–13712. [PubMed: 9593711]
34. Charlton M, Sreekumar R, Rasmussen D, et al. Apolipoprotein synthesis in nonalcoholic steatohepatitis. *Hepatology* 2002;35:898–904. [PubMed: 11915037]
35. Tabas I. Consequences of cellular cholesterol accumulation: basic concepts and physiological implications. *J Clin Invest* 2002;110:905–911. [PubMed: 12370266]
36. Soccio RE, Breslow JL. Intracellular cholesterol transport. *Arterioscler Thromb Vasc Biol* 2004;24:1150–1160. [PubMed: 15130918]
37. Mari M, Caballero F, Colell A, et al. Mitochondrial free cholesterol loading sensitizes to TNF- and Fas-mediated steatohepatitis. *Cell Metab* 2006;4:185–198. [PubMed: 16950136]
38. Shen WJ, Patel S, Natu V, et al. Interaction of hormone-sensitive lipase with steroidogenic acute regulatory protein: facilitation of cholesterol transfer in adrenal. *J Biol Chem* 2003;278:43870–43876. [PubMed: 12925534]
39. Crespo J, Cayon A, Fernandez-Gil P, Hernandez-Guerra M, et al. Gene expression of tumor necrosis factor alpha and TNF-receptors, p55 and p75, in nonalcoholic steatohepatitis patients. *Hepatology* 2001;34:1158–1163. [PubMed: 11732005]
40. Bigorgne AE, Bouchet-Delbos L, Naveau S, et al. Obesity-induced lymphocyte hyperresponsiveness to chemokines: a new mechanism of Fatty liver inflammation in obese mice. *Gastroenterology* 2008;134:1459–1469. [PubMed: 18471520]
41. Enriquez A, Leclercq I, Farrell GC, et al. Altered expression of hepatic CYP2E1 and CYP4A in obese, diabetic ob/ob mice, and fa/fa Zucker rats. *Biochem Biophys Res Commun* 1999;255:300–306. [PubMed: 10049703]
42. Emery MG, Fisher JM, Chien JY, et al. CYP2E1 activity before and after weight loss in morbidly obese subjects with nonalcoholic fatty liver disease. *Hepatology* 2003;38:428–435. [PubMed: 12883487]
43. Lieber CS. CYP2E1: from ASH to NASH. *Hepatol Res* 2004;28:1–11. [PubMed: 14734144]
44. Schulze-Osthoff K, Beyaert R, Vandevoorde V, et al. Depletion of the mitochondrial electron transport abrogates the cytotoxic and gene-inductive effects of TNF. *Embo J* 1993;12:3095–3104. [PubMed: 8344250]
45. Shimizu S, Eguchi Y, Kamiike W, et al. Involvement of ICE family proteases in apoptosis induced by reoxygenation of hypoxic hepatocytes. *Am J Physiol* 1996;271:G949–958. [PubMed: 8997238]

46. Carter-Kent C, Zein NN, Feldstein AE. Cytokines in the pathogenesis of fatty liver and disease progression to steatohepatitis: implications for treatment. *Am J Gastroenterol* 2008;103:1036–1042. [PubMed: 18177455]
47. Kremer M, Hines IN, Milton RJ, et al. Favored T helper 1 response in a mouse model of hepatosteatosis is associated with enhanced T cell-mediated hepatitis. *Hepatology* 2006;44:216–227. [PubMed: 16799967]
48. Tiegs G. Cellular and cytokine-mediated mechanisms of inflammation and its modulation in immune-mediated liver injury. *Z Gastroenterol* 2007;45:63–70. [PubMed: 17236122]
49. Biddinger SB, Hernandez-Ono A, Rask-Madsen C, et al. Hepatic insulin resistance is sufficient to produce dyslipidemia and susceptibility to atherosclerosis. *Cell Metab* 2008;7:125–134. [PubMed: 18249172]
50. Angulo P, Keach JC, Batts KP, et al. Independent predictors of liver fibrosis in patients with nonalcoholic steatohepatitis. *Hepatology* 1999;30:1356–1362. [PubMed: 10573511]
51. Chalasani N, Crabb DW, Cummings OW, et al. Does leptin play a role in the pathogenesis of human nonalcoholic steatohepatitis? *Am J Gastroenterol* 2003;98:2771–2776. [PubMed: 14687831]
52. Manco M, Marcellini M, Giannone G, et al. Correlation of Serum TNF-alpha Levels and Histologic Liver Injury Scores in Pediatric Nonalcoholic Fatty Liver Disease. *Am J Clin Pathol* 2007;127:954–960. [PubMed: 17509993]
53. Ong JP, Elariny H, Collantes R, et al. Predictors of nonalcoholic steatohepatitis and advanced fibrosis in morbidly obese patients. *Obes Surg* 2005;15:310–315. [PubMed: 15826462]

Figure 1.

Northern analysis of hepatic enzymes. Liver was extracted from fed WT and L-SACC1 mice, which had been RD- or HF-fed for 3 months, and mRNA was purified and analyzed sequentially for (A) *Cpt-1*, *Pdk4*, and *Gapdh* levels. (B) Another aliquot was analyzed for *G-6-Pase* and *Gapdh* mRNA levels. (C) and (D) Other aliquots were analyzed for *Srebp-1c* and *Pepck*, respectively, before reprobing with *Gapdh* mRNA levels. Bands were quantitated by densitometry and presented in the graph as arbitrary units. $n > 5$ from each feeding group of mice. Values are mean $\pm$ S.E. * $P < 0.05$ HF versus RD.

Figure 2.

Hepatic lipid metabolism. Liver lysates (A) from >3 mice from each of the four feeding groups (RD- and HF-WT, and RD- and HF-L-SACC1) were analyzed by immunoblotting (IB) with α -FAS, α -PPAR γ and α -NPC-1 antibodies followed by reprobing (reIB) with α -actin or α -GAPDH to normalize for the amount of protein loaded. (B) 10 μ g serum proteins from >5 mice per feeding group were analyzed by Western blotting with α -ApoB antibody to detect ApoB100 (~500kDa) and ApoB48 (~200kDa), which were quantitated by densitometry and presented in the bar graph as arbitrary units. Values are mean $\pm$ S.E. * P < 0.05 HF versus RD, $\dagger P$ < 0.05 versus WT-RD. (C) Liver sections from >5 mice of each of WT-RD (block 1), WT-HF (block 2), L-

SACC1-RD (block 3) and L-SACC1-HF (block 4) were analyzed by H&E staining for lipid accumulation.

Figure 3.

Characterization of hepatic inflammation. (A) Liver sections from >5 mice per feeding group, were analyzed by H&E staining for inflammatory cell infiltration. (B) Hepatic *Tnfa* mRNA levels from >3 mice per feeding group were determined by Northern analysis, as in Figure 1. Values are mean±S.E. * $P<0.05$ HF versus RD, † $P<0.05$ versus WT-RD. (C) Liver lysates from >3 mice per feeding group, were analyzed by Western blotting (IB) with α -phospho-NF- κ B (upper gel) and reprobing (reIB) with α -NF- κ B (lower gel). Activation of PKC ζ was similarly analyzed for the amount of phosphorylated (upper gel) relative to protein level of PKC ζ (lower gel). Bands were quantitated by densitometry and presented in the graph as arbitrary units.

Figure 4.

Immunohistochemical analysis of inflammation. Liver sections were analyzed with (A) FITC-conjugated anti-F4/80 antibody and (B) FITC-conjugated anti-CD3 and anti-CD4 antibodies. Experiments were repeated on 3 sections per mouse. Counts are mean positively stained cells per visible field $\pm$ S.E. * P <0.05 HF versus RD.

Figure 5. Nitroso-redox evaluation. (A) Liver lysates from >3 mice per feeding group were analyzed by immunoblotting (IB) sequentially with α -PPAR α , α -UCP2 and α -PGC1 α antibodies prior to reprobing (relB) with α -actin (Upper group of gels). Another aliquot was similarly analyzed with α -CYP2E1 antibody and α -actin (lower group of gels). Bands were quantitated by densitometry and presented in the bar graph as arbitrary units. (B) Liver homogenates from >5 mice per feeding group were assayed for TBARS/MDA and GSH, and serum was assayed for nitrite levels. Values are mean $\pm$ S.E. * P <0.05 HF versus RD, † P <0.05 versus WT-RD.

Figure 6.

Analysis of cell injury. (A) Liver lysates from >5 mice were analyzed by immunoblotting (IB) with α -PARP prior to reprobing with α -Tubulin. (B) Liver sections from all four feeding groups were analyzed by TUNEL (block 1–4) and H&E staining (block 5). Injured cells are emphasized by arrowheads (blocks 4 & 5).

Figure 7.

Analysis of fibrosis. (A) Liver lysates from >3 mice were analyzed by immunoblotting (IB) with α -procollagen antibody prior to reprobing (reIB) with α -tubulin. (B) Liver sections from >5 mice of each feeding group were analyzed by Sirius red staining.

Table 1

Phenotype characterization of age- and gender-matched wild type (WT) and L-SACC1 mice fed a regular-diet (RD) or high-fat diet (HF) for 3 months

	WT		L-SACC1	
	RD	HF	RD	HF
Alanine aminotransferase (μM)	42.4 $\pm$ 1.01	41.0 $\pm$ 1.15	54.2 $\pm$ 4.88 ^B	50.3 $\pm$ 4.02 ^B
Aspartate aminotransferase (μM)	140. $\pm$ 9.68	160. $\pm$ 6.27	185. $\pm$ 11.2 ^B	170. $\pm$ 7.31 ^B
Body weight-bwt (g)	20.5 $\pm$ 0.66	28.3 $\pm$ 3.10 ^A	27.1 $\pm$ 1.76 ^B	36.4 $\pm$ 1.71 ^{A,B}
Visceral fat (% bwt)	0.18 $\pm$ 0.08	5.50 $\pm$ 2.26 ^A	4.96 $\pm$ 1.33 ^B	12.4 $\pm$ 2.85 ^{A,B}
Random blood glucose (mg/dl)	67.5 $\pm$ 6.74	115. $\pm$ 9.40 ^A	108. $\pm$ 5.78 ^B	130. $\pm$ 7.50 ^{A,B}
Fasting blood glucose (mg/dl)	58.3 $\pm$ 13.1	58.0 $\pm$ 4.57	45.7 $\pm$ 4.40	87.0 $\pm$ 18.2 ^{A,B}
Serum leptin (mg/dl)	11.6 $\pm$ 1.08	33.5 $\pm$ 11.8 ^A	65.6 $\pm$ 22.2 ^B	98.0 $\pm$ 33.7 ^B
Serum insulin (pM)	73.0 $\pm$ 2.55	153. $\pm$ 22.6 ^A	153. $\pm$ 23.4 ^B	188. $\pm$ 28.2 ^B
Serum FFA (mEq/l)	0.38 $\pm$ 0.01	0.52 $\pm$ 0.07 ^A	0.68 $\pm$ 0.08 ^B	0.74 $\pm$ 0.08 ^B
Serum triglyceride (TG) (mg/dl)	40.2 $\pm$ 4.68	52.3 $\pm$ 12.4	63.4 $\pm$ 3.27 ^B	27.8 $\pm$ 3.49 ^{A,B}
Hepatic TG (mg/g protein)	23.6 $\pm$ 2.81	36.5 $\pm$ 3.07 ^A	34.2 $\pm$ 3.41 ^B	49.3 $\pm$ 3.58 ^{A,B}
Serum cholesterol (ng/dl)	61.2 $\pm$ 1.18	72.0 $\pm$ 5.36	62.7 $\pm$ 3.04	76.1 $\pm$ 9.75
Hepatic total cholesterol ($\mu\text{g}/\text{mg}$ protein)	15.4 $\pm$ 1.21	15.7 $\pm$ 0.62	13.2 $\pm$ 0.51	15.8 $\pm$ 1.27
Hepatic free cholesterol ($\mu\text{g}/\text{mg}$ protein)	11.9 $\pm$ 0.93	10.7 $\pm$ 0.50	6.83 $\pm$ 0.65 ^B	8.55 $\pm$ 0.73 ^B
Hepatic Cholesterol Esters ($\mu\text{g}/\text{mg}$ protein)	3.57 $\pm$ 0.55	4.97 $\pm$ 0.62	6.36 $\pm$ 0.46 ^B	7.28 $\pm$ 1.00 ^B
<i>In vivo</i> cholesterol synthesis ($\mu\text{mol } ^3\text{H}_2\text{O}$ incorporation/h/g)				
whole body	0.89 $\pm$ 0.10		0.69 $\pm$ 0.11	
liver	4.72 $\pm$ 0.72		9.99 $\pm$ 1.28 ^B	
heart	0.10 $\pm$ 0.10		0.06 $\pm$ 0.05	
small intestine	1.70 $\pm$ 0.16		2.07 $\pm$ 0.09	

^A $P < 0.05$ HF versus RD. Values are expressed as mean $\pm$ SEM

^B $P < 0.05$ versus WT-RD. Values are expressed as mean $\pm$ SEM