Published in final edited form as:

Hepatol Res. 2012 April ; 42(4): 412–427. doi:10.1111/j.1872-034X.2011.00934.x.

HIGH FAT DIET INDUCED HEPATIC STEATOSIS AND INSULIN RESISTANCE: ROLE OF DYSREGULATED CERAMIDE METABOLISM

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Abstract

Background—Non-alcoholic fatty liver disease (NAFLD) is an insulin resistance disease that can progress to cirrhosis or liver failure.

Hypothesis—In NAFLD, insulin resistance dysregulates lipid metabolism, increasing production of cytotoxic lipids including ceramides, which exacerbate hepatic insulin resistance and injury.

Methods—Long Evans rats were pair-fed low (LFD) or high (HFD) fat diets for 8 weeks. Livers were used to measure lipids, gene expression, insulin receptor binding, integrity of insulin signaling, and pro-inflammatory cytokines. In vitro experiments characterized effects of ceramides on Huh7 cell viability, mitochondrial function, and insulin signaling.

Results—HFD feeding caused NAFLD with peripheral and hepatic insulin resistance, increased hepatic expression of pro-ceramide genes, sphingomyelinase activity, and lipid peroxidation, and increased serum ceramide. Ceramide treatment impaired Huh7 cell viability, mitochondrial function, and insulin signaling.

Conclusions—Increased hepatic ceramide generation and release may mediate both hepatic and peripheral insulin resistance in NAFLD.

Key Terms

Insulin resistance; NASH; obesity; diabetes; ceramide; high fat diet

INTRODUCTION

Type 2 diabetes mellitus (T2DM), metabolic syndrome, non-alcoholic fatty liver disease (NAFLD), and non-alcoholic steatohepatitis (NASH) are major insulin resistance diseases whose world-wide prevalence rates are currently on the rise. T2DM is a systemic disease characterized by hyperglycemia, hyperinsulinemia, and peripheral insulin resistance, and

frequently associated with obesity. Metabolic syndrome develops in the context of visceral obesity, and is associated with T2DM, dyslipidemia and hypertension^{1, 2}. NAFLD is a spectrum of diseases, ranging from simple hepatic steatosis to NASH, and is not associated with liver infection or a history of excessive alcohol consumption¹. NAFLD is characterized by hepatocellular lipid accumulation, mainly in the form of triglycerides, resulting in greater than 5–10% increases in liver weight². NAFLD afflicts between 10 and 30 percent of the general population, and 50 to 70 percent of obese individuals^{1, 3}. Although hepatic steatosis is considered a benign and reversible condition, nearly one-third of NAFLD cases eventually progress to steatohepatitis or NASH.

NASH is a more serious disease state relative to simple hepatic steatosis due to superimposed inflammation, on-going cell death, increased pro-inflammatory cytokine activation, oxidative stress, organelle dysfunction, particularly mitochondria, and fibrogenesis^{3–5}. A diagnosis of NASH sounds alarm because of the increased risk of progressing to hepatic fibrosis, cirrhosis, and finally end-stage liver disease^{6–8}. Moreover, NASH is a risk factor for hepatocellular carcinoma^{6, 7}. The combined outcomes of cirrhosis, end-stage liver disease, and hepatocellular carcinoma have contributed to the increased rates of liver-related morbidity and mortality in Europe and North America^{9, 10}. Factors governing progression of NAFLD to NASH are not well understood, but roles have been suggested for lipotoxicity, oxidative stress, cytokine activation, and other pro-inflammatory mediators^{11, 12}.

Lipotoxicity is a state of cellular dysfunction caused by intracellular lipid overload that activates stress signaling pathways and pro-inflammatory cytokines, increases production of reactive oxygen species and toxic lipid intermediates, and inhibits mitochondrial β -oxidation^{2, 13}. Although fatty acids are widely recognized as mediators of lipotoxicity¹⁴, more recently, roles for sphingolipids and ceramides have been suggested^{15–21}. Ceramides comprise a family of lipids generated from fatty acid and sphingosine²². Ceramides are distributed in cell membranes, and in addition to their structural functions, they regulate intracellular signaling pathways that mediate growth, proliferation, motility, adhesion, differentiation, senescence, and apoptosis^{21, 23–25}. Ceramides can be generated biochemically by: 1) *de novo* synthesis through ceramide synthase and serine palmitoyltransferase mediated condensation of serine and palmitic acids²²; 2) hydrolysis of sphingomyelin through activation of neutral or acidic sphingomyelinases²⁶; or 3) degradation of complex sphingolipids and glycosphingolipids localized in late endosomes and lysosomes²⁷. Inhibition of ceramide synthesis or its accumulation prevents obesity-mediated insulin resistance^{16, 28}. Ceramides adversely alter cellular function and cause apoptosis by modulating phosphorylation states of proteins, including those that regulate insulin signaling²⁴, activating enzymes such as interleukin-1 β converting enzyme (ICE)-like proteases, which promote apoptosis²⁷, or inhibiting Akt phosphorylation and kinase activity through activation of protein phosphatase 2A²⁹.

In obesity, adipose tissue, skeletal muscle, and liver exhibit major abnormalities in sphingolipid metabolism that result in increased ceramide production, inflammation, and activation of pro-inflammatory cytokines, and impairments in glucose homeostasis and insulin responsiveness^{21, 30, 31}. In humans with NASH³², and the C57BL/6 mouse model

of diet-induced obesity with T2DM and NASH³¹, ceramide levels in adipose tissue are elevated due to increased activation of serine palmitoyltransferase, and acidic and neutral sphingomyelinases²⁷. In both genetic and diet-induced obesity, ceramide accumulation in skeletal muscle and adipose tissue may be responsible for the associated insulin resistance^{19, 31, 32}. However, NAFLD and NASH are not always associated with obesity or T2DM. Whether ceramides generated and accumulated in liver mediate hepatic insulin resistance and govern progression of NAFLD to NASH is not known. We explored this question using an experimental model of chronic high fat diet feeding.

METHODS

Animal model

Adult Long Evans male rats (n = 8 per group) were pair-fed for 8 weeks with high fat (HFD) or low fat (LFD) chow diets. The HFD supplied 60% of the kcal in fat (54% from lard, 6% from soybean oil), 20% in carbohydrates, and 20% in protein, whereas the LFD supplied 10% of the kcal in fat (4.4% from lard, 5.6% from soybean oil), 70% in carbohydrates, and 20% in protein. The HFD had 300.8 mg/kg of cholesterol, while the LFD contained 18 mg/kg of dietary cholesterol. Rats were weighed weekly, and food consumption was monitored daily. After an overnight fast, rats were sacrificed, and blood or serum was harvested to measure glucose, ALT, insulin, and lipids. Livers were removed and portions were snap-frozen and stored at -80°C, or fixed in Histochoice and embedded in paraffin. Histological sections (5 µm thick) were stained with Hematoxylin and Eosin (H&E). Our experimental protocol was approved by the Institutional Animal Care and Use Committee at Lifespan-Rhode Island hospital, and conforms to the guidelines set by the National Institute of Health.

Assessments of peripheral insulin resistance

Insulin sensitivity was evaluated at the end of the feeding period in rats anesthetized with 60 mg/kg i.p. pentobarbital. For glucose tolerance test (GTT), rats were fasted overnight fast, then administered a single i.p. injection of 2 g/kg D-glucose. For the insulin tolerance test (ITT) rats were fasted for 4 hours, then administered 0.5 U/kg i.p. human regular insulin. In both studies, glucose was measured in tail vein blood obtained at baseline, and 15, 30, 60, 120, and 180 minutes after glucose or insulin administration using a glucose meter (OneTouch Ultra, Lifespan Inc., Milpitas, CA). Responses were analyzed using the area-under-curve method, and inter-group comparisons were made using the Student T-test.

Lipid and protein adduct assays

To assess the role of adducts as mediators of oxidative stress and insulin resistance, we measured malondialdehyde (MDA) using the MDA-586 kit, and protein carbonylation using the OxyBlot assay. Carbonyl dot blot assay results were normalized to total protein spotted onto nitrocellulose membranes. Proteins were detected with SYPRO Ruby and quantified by digital imaging (Kodak Digital Science Image Station, Rochester, NY).

Lipid studies

Lipids were chloroform-methanol (2:1) extracted from fresh frozen tissue³³. Total lipid content was measured using the Nile Red microplate assay³⁴, and fluorescence (Ex 485/Em 572) was measured in a SpectraMax M5 microplate reader (Molecular Devices Corp., Sunnyvale, CA). Serum triglycerides, cholesterol, and free fatty acid levels were measured using commercially available kits, and results were normalized to liver sample weight or by protein content in the homogenate used for the extraction. Liver ceramide immunoreactivity was measured by dot blot analysis³⁵. Serum ceramide immunoreactivity was measured by direct-binding ELISA.

Gene and Protein Studies

Total RNA isolated from liver tissue using Qiazol, was reverse transcribed using random oligodeoxynucleotide primers and the AMV 1st Strand cDNA Synthesis kit. The cDNA templates were used to measure gene expression by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis using gene-specific primer pairs (Supplementary Table 1) as previously described^{33,36}.

Direct Binding Enzyme-linked Immunosorbent Assays (ELISA): Tissue homogenates were prepared in radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitors³³. Protein homogenates (50 ng/100 µl) were adsorbed to bottom well surfaces (MaxiSorp 96-well plates), then blocked with 1% BSA in phosphate buffered saline (PBS). Samples were incubated with primary antibody (0.1–0.4 µg/ml) for 1 hour at 37°C, and immunoreactivity was detected with HRP-conjugated secondary antibody and Amplex UltraRed soluble fluorophore.

Capture ELISAs measured pro-inflammatory cytokines. Capture antibodies (1 µg/ml) were adsorbed to the well bottoms as described above. 50 ng of liver homogenate were added in 100 µl, and incubated for 2 hours at room temperature. Immunoreactivity was detected with biotinylated antibodies (0.2 µg/ml for IL-1β and TNFα, and 0.5 µg/ml for IL-6), HRP-conjugated Streptavidin, and Amplex UltraRed. Fluorescence was measured (Ex 568/Em 581) in a SpectraMax M5. Binding specificity was determined from parallel control reactions with primary or secondary antibodies omitted. Immunoreactivity was normalized to protein content in parallel wells as determined with the NanoOrange Protein Quantification Kit.

Multiplex ELISAs were employed to examine the integrity of signaling through the insulin and IGF-1 receptors and downstream through IRS-1 and Akt. We used bead-based assays to measure total insulin receptor (IR), IGF-1 receptor (IGF-1R), IRS-1, Akt, proline-rich Akt substrate of 40 kDa (PRAS40), ribosomal protein S6 kinase (p70S6K), and glycogen synthase kinase 3β (GSK-3β), and phosphorylated proteins, i.e. pYpY1162/1163-IR, pYpY1135/1136-IGF-1R, pS312-IRS-1, pS473-Akt, pT246-PRAS40, pTpS421/424-p70S6K, and pS9-GSK3β, according to the manufacturer's protocol (Invitrogen, Carlsbad, CA) and as previously reported³⁷.

Receptor binding assays

Competitive equilibrium binding assays were used to examine effects of HFD feeding on insulin and IGF-I receptor binding as indices of insulin and IGF-1 resistance. To measure total binding, NP-40 lysis buffer homogenates were incubated with 50 nCi/ml (2000 Ci/mmol; 50 pM) of [¹²⁵I]-labeled insulin or IGF-I in binding buffer³⁶. Non-specific binding was measured in identical reactions containing 0.1 mM of unlabeled ligand. After 16-hours incubation at 4°C, reactions were vacuum harvested (FilterMate Harvester, PerkinElmer, Waltham, MA) onto 96-well GF/C filters pre-soaked in 0.33% polyethyleneimine. [¹²⁵I]-bound insulin or IGF-I was measured in a TopCount (Perkin Elmer, Waltham, MA). Specific binding was calculated by subtracting non-specifically bound from the total bound isotope.

Cell culture experiments

Human Huh7 hepatoma cells were maintained as previously described³⁸. 96-well cultures were treated with 0–100 μM ceramide analogs C2Cer or C6Cer, or the inactive C2DCer. After 48 hours, mitochondrial activity was measured by the MTT assay, and viability, mitochondrial mass, and ATP content were measured using the CyQuant, MitoTracker Green, and ATPLite assays, respectively^{39, 40}. Sub-confluent 6-well cultures were treated with C2Cer, C6Cer, C2DCer, or vehicle for 5 hours (less than the time needed to cause cell death), and stimulated with insulin (10 nM) or vehicle for 10 minutes. Cell homogenates were used in direct binding ELISAs (see above) to measure immunoreactivity to GSK-3β, p^{Ser9}-GSK-3β, Akt, and p^{Thr308}-Akt.

Statistical analysis

Data depicted in the graphs and tables represent the means ± S.E.M's for each group. Inter-group comparisons between the HFD and LFD groups were made using Student t-tests. Comparisons in cell culture assays were made using one- or two-way ANOVA with post hoc Dunnett's multiple comparison tests. Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA). Computer software generated P-values are shown over the graphs.

RESULTS

HFD feeding causes peripheral insulin resistance and hepatic steatosis

Chronic HFD feeding increased mean body weight (P=0.008), and elevated fasting blood glucose (P=0.01) and serum insulin (P=0.006) (Table 1). Area-under-curve analyses for changes in blood glucose or insulin over time demonstrated higher blood glucose levels in HFD relative to LFD controls. Therefore, the chronic HFD feeding caused peripheral insulin resistance and a pre-diabetic state. Although body mass was only 11.3% higher in the HFD compared to control rats, chronic HFD feeding caused striking increases in visceral fat such that all abdominal organs were encased in adipose tissue. HFD feedings did not cause hyperlipidemia; instead, the mean serum level of total lipid was reduced (Nile Red assay; P=0.02), while mean serum triglyceride and free fatty acid (data not shown) levels were similar to control (Table 1). Livers of HFD fed rats had increased mean total lipid

($P=0.0006$) and triglyceride ($P=0.001$) levels, but similar cholesterol content relative to control (Table 1). In contrast to the intact liver histology with regular chord architecture, minimal hepatocellular steatosis, and absent inflammation observed in LFD-fed controls, livers of HFD fed rats had conspicuously increased macro-vesicular and micro-vesicular steatosis with mild disorganization of hepatic cord architecture, and scattered foci of lymphomononuclear cell inflammation, but no evidence of necrosis, apoptosis, or fibrosis (Supplementary Figure 1). Therefore, in this model, chronic HFD feeding caused modest but statistically significant body weight gain, peripheral insulin resistance, and hepatic steatosis without hyperlipidemia or hepatitis.

HFD feeding and pro-inflammatory cytokines

We assessed the potential role of pro-inflammatory cytokine activation in HFD-induced NAFLD by measuring IL-1 β , TNF- α , and IL-6 mRNA by qRT-PCR analysis, and their immunoreactivities by capture ELISA (Table 2). We detected paradoxically lower levels of IL-1 β ($P=0.04$) and TNF- α ($P=0.001$) mRNA, and TNF- α ($P<0.0001$) and IL-6 ($P<0.0001$) immunoreactivity in HFD relative to LFD livers, and similar levels of IL-6 mRNA and IL-1 β immunoreactivity in the two groups. Therefore, pro-inflammatory cytokine responses in HFD rat livers were either suppressed or unchanged relative to control.

HFD causes hepatic insulin and IGF-1 resistance

We used competitive equilibrium binding assays to assess insulin resistance in liver. Since IGF-1 utilizes similar and parallel signaling pathways⁴¹ that could compensate for deficiencies in insulin signaling, we also measured IGF-1 receptor binding. Those studies revealed that chronic HFD feeding significantly reduced insulin ($P=0.0009$) and IGF-1 ($P=0.0003$) receptor binding (Figures 1A–1B). Furthermore, expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a downstream target of insulin/IGF signaling, was reduced by HFD feeding ($P<0.0001$; Figure 1C), whereas β -actin was not (Figure 1D). Livers from HFD fed rats also had reduced expression of insulin ($P=0.026$) and IGF-1 ($P=0.009$) receptors, and Akt ($P=0.014$) relative to control (Table 3), and increased levels of p70S6K ($P=0.038$) and pSer³⁰⁷-IRS-1 ($P=0.022$). The latter has an inhibitory effect on insulin signal transduction⁴². On the other hand, immunoreactivity corresponding to most of the other phosphorylated molecules in the insulin/IGF-1 signaling cascade were not altered by chronic HFD feeding.

Hepatic steatosis and pro-ceramide gene expression

We next examined the potential role of ceramides as mediators of hepatic insulin/IGF-1 resistance because: 1) ceramides are bioactive sphingolipids that regulate intracellular signaling and have a demonstrated role in promoting insulin resistance^{21, 22, 43}; 2) previous studies of obesity or HFD feeding revealed increased ceramide levels in skeletal muscle and adipose tissue, both of which are highly insulin-responsive^{24, 31, 44}; and 3) de novo synthesis of ceramides is triggered by increased availability of fatty acids²³ (See Table 4). Our studies included qRT-PCR to measure expression of genes that regulate ceramide biosynthesis and degradation, enzymatic assays of neutral and acid sphingomyelinase activities, and ELISAs or dot blots to assay ceramide immunoreactivity in serum and liver.

Exploratory qRT-PCR analyses demonstrated that Ceramide Synthase (Cer) 1, 2 and 4, acidic and neutral isoforms of sphingomyelin phosphodiesterase (sphingo-myelinase; SMPD1 and SMPD3), UDP glucose ceramide glycosyltransferase (UGCG), serine palmitoyl transferase (SPTLC) 1 and 2, and ceramidase (CERD) 2 and 3 were abundantly expressed in liver. CER 1, 2, and 4, and SPTLC2 mRNAs were all significantly increased in HFD relative to LFD livers (Figure 2). In contrast, UGCG and SPTLC1 were expressed at similar levels in the two groups. Among genes involved in lipid degradation, CERD2, CERD3, SMPD1, and SMPD3 genes were all expressed at higher in HFD compared with LFD livers (Figures 3A–3D). Enzymatic assays demonstrated higher levels of acidic sphingomyelinase ($P=0.01$), but not neutral sphingomyelinase activity in the HFD group (Figures 3E–3F). Therefore, chronic HFD feeding significantly increased hepatic expression of genes that generate ceramides by N-acylation of sphingosine through the actions of ceramide synthases²³, hydrolysis of sphingomyelin via sphingomyelinases²⁷, and condensation of palmitic acid and serine through the catalytic action of SPTLC2. However, HFD feeding did not increase the non-regulated SPTLC1 subunit, or UGCG, the rate-limiting enzyme glucosylceramide, which is needed for de novo ceramide synthesis, and is dysregulated in humans with NAFLD⁴⁵. Finally, we measured significantly higher ceramide levels in HFD serum, and although ceramide levels were also higher in HFD livers, the difference from control did not reach statistical significance (Figures 3G–3H).

Chronic HFD feeding increases oxidative stress and lipid adducts

Ceramides promote insulin resistance and oxidative stress^{16–21, 46, 47}. Persistent oxidative stress increases formation and accumulation of lipid, protein, and DNA adducts, which impair cellular functions and promote cellular degeneration and death. To determine the degree to which HFD-mediated hepatic insulin resistance, pro-ceramide gene up-regulation, and increased ceramide production were associated with increased oxidative stress and lipid peroxidation, we examined: 1) malondialdehyde (MDA); 2) protein carbonyls; and 3) 3-nitrotyrosine (N-Tyr) in liver. The mean MDA level, reflecting toxic aldehyde generated by reactive oxygen species attacks on lipids⁴⁸, was significantly higher in HFD compared with the LFD-fed controls ($P=0.03$; Figure 4A). Despite significantly higher mean hepatic triglyceride and MDA levels in the HFD-fed group, the calculated mean MDA/Triglyceride ratio was significantly lower in the HFD (0.128 ± 0.01) compared with the LFD (0.261 ± 0.02) ($P=0.003$) group, indicating that other factors in addition to hepatic triglyceride accumulation, govern formation of lipid adducts. The mean protein carbonyl levels were similar in livers of HFD- and LFD-fed rats (Figure 4B). NTyR, which marks protein nitrotyrosilation caused by peroxynitrite attack, and could interfere with protein phosphorylation/dephosphorylation signaling and protein functions^{49, 50}, was significantly increased in livers of HFD- relative to LFD-fed control rats ($P=0.02$; Figure 4C).

Ceramide-mediated hepatotoxicity and insulin resistance

Dose-response studies of mitochondrial function using the MTT assay demonstrated relative resistance of Huh7 cells to low concentrations ($<10 \mu\text{M}$) of C2, C6, or C2D (x48 h), but marked sensitivity to higher concentrations of C2 ceramide (Figure 5A). Further analyses of viability, mitochondrial mass and mitochondrial function were performed on cells treated with $50 \mu\text{M}$ ceramide for 48 h. Those studies demonstrated significantly reduced viability in

C2Cer $\gg$ C6Cer treated relative to vehicle or C2DCer treated cultures (Figure 5B). Analysis of mitochondrial mass using the Mitotracker green (MTG) fluorescence assay revealed significantly increased MTG fluorescence, reflecting mitochondrial proliferation in C2Cer-treated relative to C6C, C2DCer, and vehicle-treated cultures (Figure 5C). Finally, assessments of viability with the CyQuant assay, and mitochondrial function using the ATPlite assay, revealed that C2Cer and C6Cer exposures significantly reduced viability and ATP production (Figure 5D).

Ceramides antagonize insulin signaling by activating protein phosphatase PP2A, resulting in dephosphorylation of PKB/AKT²⁰. To confirm that ceramides cause insulin resistance, we exposed Huh7 cells to C2Cer, C6Cer, C2DCer, or vehicle for 5 hours (much shorter than the 24–48 hours required to cause cytotoxicity or mitochondrial dysfunction), after which the cells were stimulated with insulin or vehicle for 10 minutes. GSK-3 β , phospho-GSK-3 β (Ser9) AKT and phospho-AKT (Thr308) levels were measured by direct binding ELISA. The mean levels and percentage increases in GSK-3 β were significantly higher in insulin-stimulated C2Cer and C6Cer treated relative to their corresponding un-stimulated cultures (Figures 6A, 6B). Although the mean levels of phospho-GSK-3 β were not significantly altered by insulin stimulation, C6Cer-treated cells had significant reductions in the relative levels of phospho-GSK-3 β in paired assays (Figures 6C, 6D). Total Akt was significantly higher in insulin-stimulated C2Cer-treated relative to unstimulated cultures, and the mean percentage increases in insulin-stimulated total Akt were significantly higher in both C2Cer and C6Cer treated relative to vehicle-treated cells (Figures 6E, 6F). Finally, insulin stimulation significantly increased the mean levels of phospho-Akt in vehicle, C2DCer, and C2Cer, but not in C6Cer treated cells (Figure 6G); instead, insulin-stimulated C6Cer-treated cells had significant mean percentage reductions in phospho-Akt relative to vehicle-treated cells (Figure 6H).

DISCUSSION

NAFLD and NASH, like T2DM and metabolic syndrome, are insulin resistance diseases, and they frequently overlap with T2DM or metabolic syndrome due to high co-morbidity rates with obesity. When associated with T2DM or metabolic syndrome, hepatic insulin resistance could be mediated by pro-inflammatory cytokines and oxidative stress. However, in the absence of obesity, T2DM, or metabolic syndrome, which factors promote hepatic insulin resistance in NAFLD? One consideration is that steatosis-induced modifications of cell membrane lipid composition may impair insulin sensitivity^{51–54}. Herein, we investigated the potential role of toxic lipids, particularly ceramides, because, in both genetic and DIO models, ceramides accumulate in skeletal muscle and adipose tissue, and they inhibit insulin signaling^{24, 31, 55–57}.

In our model of chronic HFD feeding, modest (11.3%) but significantly increased mean body weight was associated with visceral obesity, peripheral insulin resistance, and pre-T2DM. In contrast to related studies⁵⁸, the HFD-fed rats did not have hyperlipidemia or metabolic syndrome. Histopathologic, biochemical, and molecular studies confirmed the presence of significant hepatic steatosis with minimal inflammation, apoptosis, necrosis, and pro-inflammatory cytokine activation. Transition from simple hepatic steatosis to NASH has

been linked to pro-inflammatory cytokine activation^{11, 12, 59}. Correspondingly, in our model, HFD produced NAFLD instead of NASH, perhaps due to the absence of inflammatory mediators in the steatotic livers. Although hyperlipidemia is considered to be risk factor for NAFLD¹, we found that NAFLD can occur despite a favorable serum lipid profile. While elevated ALT levels and peripheral insulin resistance may suffice to suspect a diagnosis of NAFLD, additional serum biomarkers are needed to detect and monitor NAFLD, and prevent its progression to NASH.

Hepatic insulin resistance in our model of NAFLD was associated with reduced insulin receptor binding, insulin receptor and Akt immunoreactivity, and GAPDH expression, and increased p^{S307}-IRS-1. GAPDH is an important insulin-responsive gene⁶⁰, and phosphorylation of IRS-1 on S307 inhibits downstream signaling through IRS-1⁴². In addition, we detected NAFLD-associated impairments in IGF-1 signaling manifested by reduced binding to the IGF-1 receptor and reduced IGF-1 receptor expression. In addition, we detected significantly increased levels of p^{TpS421/424}-p70S6K in HFD-exposed livers, consistent with previous studies of diet-induced obesity^{61, 62}. The finding of similar mean levels of pYpY1162/1163-IR, pYpY1135/1136-IGF-1R, p^{S312}-IRS-1, p^{S473}-Akt, p^{T246}-PRAS40, and p^{S9}-GSK3 β in livers of HFD and LFD fed rats indicates that, despite impairments in upstream signaling at the receptor level, compensatory measures may have minimized the net impairments in insulin/IGF-1 signaling. In this regard, the increased levels of p^{TpS421/424}-p70S6K in HFD-exposed livers could reflect enhanced insulin/IGF-1 stimulated growth and metabolic functions mediated by increased signaling through PI3 kinase-Akt⁶³. Alternatively, the finding could represent activation of negative feedback on insulin signaling^{61, 62}, and thereby account for the increased serine phosphorylation of IRS-1 observed herein.

Although hepatic steatosis generally reflects storage of triglycerides in hepatocytes⁶⁴, there is growing evidence that other lipid species also accumulate⁶⁵ in relation to NAFLD/NASH. We interrogated whether the insulin resistance in experimental NAFLD was mediated by dysregulated sphingolipid metabolism, focusing on the role of ceramides. Ceramides are of interest because they: 1) have anti-growth properties and promote apoptosis and senescence; 2) activate protein phosphatases such as PP2A and PP1, which negatively regulate insulin signaling mechanisms²⁵; 3) induce insulin resistance by antagonizing PI3K/AKT^{17, 18, 24}; and 4) accumulate in insulin responsive tissues such as adipose tissue and skeletal muscle in diet-induced and genetic models of obesity/T2DM^{24, 31, 66}, and in obese humans^{32, 44}.

We found that NAFLD is associated with increased expression of multiple pro-ceramide genes, including those mediating de novo biosynthesis, hydrolysis of sphingomyelin, and the activity of ceramide synthases, consistent with recent findings⁶⁷. These changes were associated with increased sphingomyelinase activity in liver, and ceramide levels in sera of HFD-fed rats, which could lead to worsening of hepatic and peripheral insulin resistance. Although there was a trend toward higher hepatic ceramide levels in the HFD group, the failure to detect significantly increased levels of ceramide in liver could have been due to rapid transport of ceramide into serum and subsequent trafficking to other tissues (see supplementary Figure 2) where they could exert toxic and degenerative effects, including

insulin resistance^{37, 68}. Alternatively, the increased activation of ceramidases may have reduced net ceramide levels in liver. However, ceramidases convert ceramide to sphingosine, and sphingosine is cytotoxic and causes apoptosis²³. Sphingosine also antagonizes insulin signaling, glucose transport, and lipogenesis at the post receptor level^{69, 70}. On the other hand, over-expression of acid ceramidase, which degrades ceramide to sphingosine and fatty acids, protects cells from the inhibitory effects of palmitate and other long chain saturated FFAs on insulin signaling²⁸. In palmitate treated cells, acid ceramidase over-expression decreases ceramide, and increases its breakdown product sphingosine. These responses can restore insulin responsiveness in FA expose cells, and suggest that ceramides rather than sphingosine mediate insulin resistance.

In vitro experiments tested the hypothesis that bioactive toxic ceramides can mediate many of the adverse effects of chronic HFD feeding, including cytotoxicity, mitochondrial dysfunction, ATP deficiency, and impaired insulin signaling through Akt and GSK-3 β . The additional findings that ceramide exposure and insulin stimulation can modulate levels of total Akt or GSK-3 β are consistent with emerging evidence that, besides phosphorylation, signaling through these molecules can be regulated by changes in their intracellular sequestration and accumulation^{71, 72}. The discrepancy with respect to ceramide inhibition of Akt phosphorylation in vitro but not in vivo could have been due to the absence of adaptive responses in acute compared with chronic ceramide exposure.

Roles for ceramide metabolites, e.g. glycosphingolipids, as mediators of insulin resistance and cytotoxicity have been demonstrated in experimental obesity and chronic HFD feeding^{55, 57, 73}. Chemical inhibition of glucosylceramide synthase in ob/ob mice, Zucker diabetic fatty rats, and 3T3-L1 adipocyte cultures reduces or reverses insulin signaling abnormalities, insulin resistance, and pro-inflammatory cytokine activation^{55, 57}. Herein, we measured UDP-glucose ceramide glucosyltransferase (UGCG) by qRT-PCR and found no significant elevation of its mRNA in the HFD group. Although UGCG expression is increased in NASH, our finding of non-elevated levels of UGCG suggests that glycosphingolipids may mediate transition from NAFLD to NASH. In addition, there is growing evidence that various sphingolipids incorporated into cell membrane lipid rafts which contain receptor tyrosine kinases, mediate insulin resistance and therefore represent potential pharmacotherapeutic targets¹³. For example, sphingoid base 1-phosphate promotes oxidative stress, pro-apoptotic signaling, and cytotoxicity⁷⁴. Finally, ceramides themselves can cause insulin resistance, as demonstrated here and elsewhere^{30, 32, 68, 75–77}. Conceivably, a better understanding of the role of ceramides as mediators of insulin resistance will require characterization of the profiles associated with insulin resistance, oxidative stress, and cytotoxicity, and therefore mediating transition from NAFLD to NASH.

Ceramides cause cell death by activating pro-inflammatory cytokines and promoting oxidative stress. In our model of NAFLD, although we found no evidence of pro-inflammatory cytokine activation, several indices of oxidative stress including lipid peroxidation and peroxynitrite were elevated in the livers. Oxidative stress impairs insulin signaling, exacerbates insulin resistance⁴⁹, and promotes formation of DNA, protein and lipid adducts, which interfere with vital functions. Since stress induced adducts can bolster

the transition from simple steatosis to NASH ⁷⁸, in our model, the failure to progress from NAFLD to NASH may have been due to the modest accumulations of protein and lipid adducts.

In conclusion, chronic HFD feeding caused NAFLD with peripheral as well as hepatic insulin resistance, but with minimal evidence of inflammation. In vitro experiments support our hypothesis that increased activation of pro-ceramide mechanisms with attendant elevated levels of ceramides and other toxic lipids, e.g. sphingosine and glycosphingolipids in liver are key factors mediating hepatic insulin resistance in NAFLD. The data suggest that inflammation, cytokine activation, and increased stress may be required to promote transition from NAFLD to NASH. Given the elevated serum ceramide but not triglyceride levels in this model of chronic HFD with viscera-restricted obesity, we propose that serum ceramide levels may serve as peripheral non-invasive biomarkers of NAFLD. Therapeutic measures to inhibit hepatic production of ceramides and other toxic lipids may reduce the severity and progression of NAFLD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported by AA-11431, AA-12908, and AA16126 from the National Institutes of Health

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Figure 1.

Chronic HFD feeding impairs insulin and IGF-1 receptor binding in liver. Competitive equilibrium binding studies were performed with 8 liver protein homogenate samples per group from LFD or HFD fed rats. Reactions were incubated with 50 nCi/ml of [125 I]-labeled insulin or IGF-1 (2000 Ci/mmol; 50 pM) in the presence or absence of 0.1 μ M unlabeled ligand. Bound ligand was harvested onto 96-wells GF/C plates and measured in a TopCount machine. Specific binding was calculated by subtracting non-specifically bound from the total bound isotope. Box plots depict median (horizontal bar) $\pm$ 95% C.I.L. (upper and lower borders of boxes), and range (whiskers) of specific binding for (A) insulin and (B) IGF-1. (C) Insulin resistance was further assessed by measuring GAPDH immunoreactivity (ELISA). (D) β -actin was measured as a negative control (ELISA). Inter-group statistical comparisons were made using Student T-tests. Significant P-values are shown within the panels. RFU=relative fluorescence units.

Figure 2.

Effects of chronic HFD feeding on expression of pro-ceramide genes-biosynthetic pathways. RNA extracted from HFD and LFD rat livers (N=8/group) was used to measure gene expression by qRT-PCR analysis with gene-specific primer pairs (See Methods and Supplementary Table 1). Gene expression was normalized to 18S rRNA. Box plots depict relative levels of gene expression for (A–C) Ceramide synthases (CER) 1, 2, 4, (D, E) serine palmitoyl transferases (SPTLC), and (F) UDP glucose ceramide glycosyltransferase (UGCG). Inter-group comparisons were made using Student t-tests. Significant P-values are shown over the graphs.

Figure 3.

Effects of chronic HFD on expression of (A–D) ceramide-related genes and (E, F) enzymatic activity-degradation pathways from sphingomyelin, and (G, H) ceramide levels in liver and serum. HFD and LFD liver RNA was used in qRT-PCR analyses (N=8/group) (See Methods and Supplementary Table 1). (A–D) Ceramidase (CERD) and (C, D) sphingomyelinase mRNA transcript levels were normalized to 18S rRNA. (E) Acidic and (F) neutral sphingomyelinase activities were measured using a commercial assay. Ceramide immunoreactivity was measured in (G) liver by dot-blot analysis, and (H) serum by ELISA. Box plots show data corresponding to (A–D) relative levels of gene expression, (E, F) sphingomyelinase activity, and (G, H) immunoreactivity. Inter-group comparisons were made using Student t-tests. Significant P-values are shown above the bars. RLU=relative luminescence units; D.U.= densitometry units.

Figure 4.

Chronic HFD causes hepatic oxidative stress. (A) Malondialdehyde (MDA), (B) total carbonylated proteins, and (C) 3-nitrotyrosine (N-TYR) immunoreactivity were assayed in fresh frozen liver tissue as described (see Methods) and inter-group comparisons are shown with box plots. Inter-group comparisons were made with Student t-tests. Significant P-values are indicated within each panel. (D.U.=densitometry units; FLU= Fluorescence light units)

Figure 5.

In vitro ceramide exposure impairs mitochondrial function and causes cytotoxicity. Huh7 hepatic cells were exposed to different concentrations of C2Cer, C6Cer, C2DCer (inactive), or vehicle (0 point) for 48 hours. (A) Ceramide dose-effects on cell density and mitochondrial function using the MTT assay with absorbances measured at 540 nm. (B–D) Cells were exposed to 50 μ M ceramide for 48 hours, and then used to measure (B) viability with the Cyquant (CYQ) assay, (C) mitochondrial mass by the Mitotracker Green (MTG) fluorescence, and (D) ATP using the ATPlite assay (RLU=relative light units). Box plots depict results from 8 replicate 96-well cultures. Absorbances, fluorescence intensity, and luminescence were measured in a Spectramax M5 microplate reader. Data were analyzed by one-way ANOVA with post hoc Dunnett tests. Significant P-values are indicated within each panel.

Figure 6.

Short-term ceramide exposure impairs insulin signaling. Huh7 cells were treated with Vehicle, C2D (inactive), C2 or C6 (50 μM) ceramide for 5 hours, then stimulated with vehicle (-) or 10 nM insulin (+) for 10 minutes. Immunoreactivity to (A) total GSK-3β, (C) p^{Ser9}-GSK-3β (E) total AKT, or (G) p^{Thr308}-AKT was measured by ELISA using HRP-conjugated secondary antibody and the Amplex UltraRed soluble fluorophore. Fluorescence light units (FLU) were measured (Ex 568 nm/Em 581 nm) in a Spectramax M5. (B, D, F, H) Percentage increases in insulin-stimulated over basal immunoreactivity were calculated, and results are depicted using box plots. Inter-group comparisons were made using (A, C, E, G) two-way or (B, D, F, H) one-way ANOVAs with the post hoc Dunnett tests. Significant P-values are shown within each panel.

Table 1**High Fat Diet Feeding Causes Type 2 Diabetes and Hepatic Steatosis**

	Low Fat Diet	High Fat Diet	P-Value
Body Weight (g)	391.8 ± 11.4	441.9 ± 13.2	0.008
Blood Glucose (mg/dl)	108.2 ± 3.2	121.2 ± 3.5	0.01
Serum Insulin (ng/ml)	0.41 ± 0.10	1.23 ± 0.26	0.006
GTT (mg/dl-AUC)	33474 ± 3212	44421 ± 2684	0.0129
ITT (ng/ml-AUC)	20953 ± 882	27745 ± 1125	0.0004
<u>Serum Lipids and ALT</u>			
Nile Red (FLU/μl)	246.0 ± 31.3	174.4 ± 7.03	0.02
Triglycerides (mg/ml)	0.36 ± 0.05	0.30 ± 0.06	
ALT (U/l)	19.27 ± 1.06	29.43 ± 1.6	0.0001
<u>Hepatic Lipids</u>			
Nile Red (FLU/mg)	103.2 ± 10.5	205.0 ± 21.1	0.0006
Triglycerides (μM/g)	28.8 ± 3.8	54.4 ± 4.8	0.001
Cholesterol (μg/mg)	125.3 ± 9.7	115.6 ± 0.5	

Adult male Long Evans rats were maintained on a high fat (60% of calories) or low fat (10% of calories) diet for 8 weeks (N=8 per group). After an overnight fast, rats were sacrificed and liver and blood were harvested immediately. Blood or serum was used to measure glucose, insulin, alanine aminotransferase (ALT), total lipid (Nile Red assay), triglycerides, cholesterol, and free fatty acid levels. In addition, peripheral insulin resistance was assessed with glucose (GTT) and insulin (ITT) tolerance tests (see Methods), and Area-under-curve analysis was used for inter-group comparisons, and performed with blood glucose levels measured from 0–180 minutes after bolus injection of glucose (GTT) or insulin (ITT). Liver lipid extracts were used to measure lipid content, triglycerides, and cholesterol and normalized by protein content in the homogenate used for the extraction. Data depict the mean ± S.E.M. results for each group. Inter-group comparisons were made using Student T-tests, and significant P-values are listed.

Table 2**Effect of High Fat Diet on Hepatic Cytokine Expression**

Hepatic Cytokines	Low Fat Diet	High Fat Diet	P-Value
mRNA/18S rRNA*			
IL-1 β	0.048 $\pm$ 0.004	0.034 $\pm$ 0.005	0.04
TNF- α	0.020 $\pm$ 0.002	0.011 $\pm$ 0.001	0.001
IL-6	0.007 $\pm$ 0.001	0.005 $\pm$ 0.0005	
ELISA (RFU)			
IL-1 β	756.6 $\pm$ 12.8	774.2 $\pm$ 13.8	
TNF- α	974.7 $\pm$ 27.7	780.7 $\pm$ 19.1	<0.0001
IL-6	1076.0 $\pm$ 19.9	881.2 $\pm$ 12.4	<0.0001

Adult male Long Evans rats were maintained on a high fat (60% of calories) or low fat (10% of calories) diet for 8 weeks (N=8 per group). Livers were harvested immediately after sacrifice. The mRNA levels of IL-1 β , TNF- α , IL-6 were measured by qRT-PCR analysis, and corresponding immunoreactivity was assessed by capture ELISA with Amplex UltraRed fluorescence detection. Values represent relative fluorescence light units (RFU, Ex 568/Em 581). The mRNA results are normalized to 18S rRNA measured in the same samples (see Methods). Data depict the mean $\pm$ S.E.M. results for each group. Inter-group comparisons were made using Student T-tests, and significant P-values are listed.

Table 3Insulin/IGF-1 Pathway Analysis Through IRS-1, Akt and GSK-3 β

Total Protein* (RFU)	Low Fat Diet	High Fat Diet	Significance
Insulin Receptor	343.3 + 11.49	297 + 16.81	P=0.026
IGF-1 Receptor	282.4 + 11.74	221.3 + 14.90	P=0.009
IRS-1	501.3 + 11.58	473.3 + 26.52	
Akt	5092 + 213.2	4041 + 264.40	P=0.014
PRAS40	439.3 +14.56	437.6 + 17.66	
p70S6K	427.6 +28.01	398.5 +24.53	
GSK-3 β	1189 + 179.3	949.5 + 143.9	

Phospho-Protein* (RFU)	Low Fat Diet	High Fat Diet	Significance
pYpY1162/1163-IR	299.6 + 34.07	297.1 + 33.04	
pYpY1135/1136-IGF-1 R	130.9 + 12.08	122.3 + 6.15	
pS312-IRS-1	219.8 + 10.51	202.5 + 6.33	
pS473-Akt	1104 + 110.9	1285 + 166.60	
pT246-PRAS40	228.4 + 19.32	250.2 + 14.27	
pTpS421/424-p70S6K	138.2 + 28.84	275.9 + 51.18	P=0.038
pS9-GSK3 β	273.8 + 7.71	296.8 + 20.46	
pS307-IRS1	8.204 + 0.26	8.781 + 0.09	P=0.022

Adult male Long Evans rats were maintained on a high fat (60% of calories) or low fat (10% of calories) diet for 8 weeks (N=8 per group). The total and phosphorylated levels of key components of the insulin signaling pathway were measured in liver tissue homogenates via bead-based multiplex ELISA. Values represent relative fluorescence light units (RFU) after normalization for the amount of protein used for the assay. Data depict the mean $\pm$ S.E.M. results for each group. Inter-group comparisons were made using Student T-tests, and significant P-values are listed.

* IR=insulin receptor, IGF-1R=IGF-1 receptor; IRS-1=insulin receptor substrate, type 1; PRAS40=Proline-rich Akt substrate of 40 kDa; p70S6K=70 kDa ribosomal protein S6 kinase; GSK-3 β =glycogen synthase kinase-3 β

Table 4**Ceramide-Related Genes and Their Functions**

Abbreviation	Full name	Function
CER1	ceramide synthase 1 (Lass1)	N-acylation of sphingoid long chain base with fatty acyl-CoAs (mainly C18) to form ceramide [#]
CER2	ceramide synthase 2 (Lass2)	N-acylation of sphingoid long chain base with fatty acyl-CoAs (mainly C20-26) to form ceramide [#]
CER4	ceramide synthase 4 (Lass4)	N-acylation of sphingoid long chain base with fatty acyl-CoAs (mainly C24) to form ceramide [#]
SMPD1	sphingomyelin phosphodiesterase 1, acid lysosomal	Synthesis of ceramide from sphingomyelin (lysosomal)
SMPD3	sphingomyelin phosphodiesterase 3, neutral	Synthesis of ceramide from sphingomyelin (plasma membrane)
UGCG	UDP-glucose ceramide glucosyltransferase	Synthesis of glucosyl ceramide
CERD1	N-acylsphingosine amidohydrolase (acid ceramidase)	Hydrolysis of ceramide to sphingosine (lysosomal) ^{\$}
CERD2	N-acylsphingosine amidohydrolase 2 (neutral ceramidase)	Hydrolysis of ceramide to sphingosine (plasma membrane) ^{\$}
CERD3	N-acylsphingosine amidohydrolase 3-like (alkaline ceramidase 2)	Hydrolysis of ceramide to sphingosine (Golgi apparatus) ^{\$}
SPTLC	serine palmitoyltransferase,	Catalyses the first step of de novo ceramide synthesis
N-SMase	sphingomyelin phosphodiesterase 3, neutral	Synthesis of ceramide from sphingomyelin
A-SMase	sphingomyelin phosphodiesterase 1, acid lysosomal	Synthesis of ceramide from sphingomyelin

^{\$} Ref: Mao C. Ceramidases: regulators of cellular responses mediated by ceramide, sphingosine and sphingosine 1-phosphate. *Biochimica et biophysica Acta* (2008) 424–434

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