Published in final edited form as:

Dig Dis Sci. 2008 October ; 53(10): 2761–2774. doi:10.1007/s10620-007-0193-7.

The Transition from Fatty Liver to NASH Associates with SAME Depletion in db/db Mice Fed a Methionine Choline-Deficient Diet

Matthew Wortham, Lin He, Maxwell Gyamfi, Bryan L. Copple, and Yu-Jui Yvonne Wan

Department of Pharmacology, Toxicology, and Therapeutics, University of Kansas Medical Center, Kansas City, KS 66160, USA

Yu-Jui Yvonne Wan: ywan@kumc.edu

Abstract

Nonalcoholic fatty liver disease (NAFLD) is highly prevalent in the Western population. By mechanisms that are not completely understood, this disease may progress to nonalcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). db/db mice spontaneously develop hepatic steatosis, which progresses to NASH when these mice are fed a methionine choline-deficient (MCD) diet. The goal of our studies was to identify lipid and methionine metabolism pathways affected by MCD feeding to determine potential causal events leading to the development of NASH from benign steatosis. db/db mice fed the MCD diet for 2 weeks exhibited signs of incipient NASH development such as upregulated cytokines and chemokines. At this time point, MCD diet feeding caused S-adenosylmethionine (SAME) depletion in db/db mice, while wild-type mice on the same diet retained hepatic SAME levels. SAME depletion exerts pleiotropic effects upon liver homeostasis and is commonly associated with a variety of liver insults such as thioacetamide, CCL₄, and alcohol treatment; thus, SAME depletion may serve as the second hit in NASH development. It is possible that differences in hepatic lipid and/or methionine metabolism between wild-type and db/db mice underlay the differential maintenance of SAME levels during methionine and choline restriction. Indeed, db/db mice exhibited inhibited lipid oxidation pathways, which may be a priming factor for NASH development, and db/db mice fed the MCD diet had differential methionine adenosyltransferase (MAT) expression. The occurrence of SAME depletion at this early, benign stage of NASH development in db/db mice with fatty liver suggests that SAME supplementation may be (A) targeted to individuals susceptible to NASH (i.e., NAFLD patients) and (B) preventative of NASH before substantial liver injury has occurred.

Keywords

Fatty liver; Hepatitis; Methionine adenosyltransferase; Methylation

Introduction

Excessive food intake and sedentary lifestyles have led to an epidemic of the metabolic syndrome, or an energy imbalance resulting in hypertension, insulin resistance, and dyslipidemia. In addition to the classical cardiovascular and insulin response defects caused by the metabolic syndrome, obese individuals have a higher prevalence of fatty liver as well as nonalcoholic steatohepatitis (NASH) (i.e., fatty liver with inflammation), which is a progressive disease leading to fibrosis, cirrhosis, and finally hepatocellular carcinoma [1]. Nonalcoholic fatty liver disease (NAFLD) is highly prevalent in Western populations. Recent studies suggest that this disease may occur at a frequency of 70% in obese individuals and 35% in lean individuals [2]. The current model of pathogenesis from healthy liver to NASH suggests a two-hit progression. First, insulin resistance causes lipid accumulation in hepatocytes (first hit). Secondly, it is proposed that cellular insults such as oxidative stress, lipid peroxidation, direct lipid toxicity, mitochondrial dysfunction, and/or infection causes hepatic inflammation (second hit), resulting in NASH [3]. Obesity has been associated with chronic systemic inflammation resulting in elevated serum cytokines, such as tumor necrosis factor α (TNF α) [4], suggesting that fatty liver could naturally progress to NASH in obese individuals. Understanding the causes of NASH in high-risk individuals (i.e., those with fatty liver) would be central for development of NASH-preventive regimens.

db/db mice are obese and insulin resistant due to a naturally occurring mutation in the leptin receptor (Ob-Rb) gene resulting in a splice defect that truncates the receptor to the length of the short-form (Ob-Ra) leptin receptor, ablating central leptin signaling in the hypothalamus [5]. Aberrant leptin signaling results in energy imbalance; thus, db/db mice develop macrovesicular hepatic steatosis under normal conditions and readily exhibit symptoms of NASH upon induction of a second hit, for example, by feeding of an MCD diet [6, 7]. MCD diet feeding mimics NASH pathology in man; however, methionine and choline deficiency does not likely reflect a dietary deficiency that would occur in obese patients. db/db mice fed an MCD diet for 4 weeks exhibit severe inflammation and fibrosis, whereas wild-type C57/BL6 mice fed the MCD diet for 4 weeks develop macrovesicular steatosis with moderate inflammation but exhibit negligible fibrosis [7] (See Table 1). After 10 weeks of MCD diet feeding, C57/BL6 mice develop early-stage fibrosis [8].

Abnormalities of lipid metabolism induced by nonfunctional leptin signaling clearly plays a role in steatosis induced in db/db mice; however, the mutation itself is not sufficient to induce NASH [7]. The original rationale for MCD diet feeding was to restrict availability of essential components of phosphatidylcholine synthesis, which is necessary for lipoprotein formation and thus lipid export from the liver [6]. However, because lipid accumulation alone is not sufficient to induce NASH in this model, lipid sequestration induced by MCD diet feeding is probably only one of multiple pathways causing the second hit in this model. Indeed, in wild-type rats fed an MCD diet, which develop steatosis and then NASH, methionine supplementation is sufficient to prevent NASH but not steatosis, suggesting that choline restriction alone is sufficient for induction of steatosis [9]. Thus it is possible that in db/db mice fed an MCD diet, abnormal methionine metabolism induced by the diet

synergizes with abnormal lipid metabolism inherent in db/db mice to promote development of NASH.

Methionine is the precursor of SAMe, the principal biological methyl donor involved in synthesis of lipoproteins and the antioxidant glutathione (GSH). Methylation pathways utilizing SAMe also affect membrane fluidity, reactive oxygen species (ROS) generation, cellular differentiation, and the balance between proliferation and apoptosis [10]. These pathways may account for MCD-induced effects besides lipid sequestration that contribute to NASH development. In wild-type rats, methionine restriction during MCD diet feeding for 5 weeks (until the onset of fibrosis) has been shown to promote SAMe depletion [9], and SAMe supplementation coincident with MCD feeding reduces the severity of NASH [11]. In animal models, SAMe depletion is common among various chemical-induced liver injuries, whereas in many such cases SAMe supplementation attenuates liver injury [12–19]. Thus, SAMe supplementation may have potential for clinical application in patients with a predisposition to NASH.

SAMe depletion could result from decreased synthesis or increased utilization. SAMe is principally utilized in a variety of transmethylation pathways in the liver, notably in phosphatidylcholine and glutathione synthesis. SAMe is synthesized from methionine and ATP by methionine adenosyltransferases MAT1/III (liver specific) and MAT2 (not liver specific, inhibited by high levels of SAMe) [10]. Reduction of liver-specific MAT1/III activity and/or induction of non-liver-specific MAT2 activity is a common biomarker of many chemically induced liver injuries, such as thioacetamide [14], CCL₄ [20], and ethanol treatment [16]. Additionally, patients with cirrhosis caused by viral hepatitis demonstrate reduced total MAT activity [21] and reduced mRNA levels of MAT1A [22]. Thus, abnormal MAT isoform activities in the liver could be a cause of reduced SAMe levels.

The association of SAMe depletion with specific events in the progression of liver disease (i.e., steatosis, early or late NASH) would be informative regarding the potential for therapeutic intervention in patients with various degrees of fatty liver and NASH progression. Additionally, identification of the causes for SAMe depletion in MCD-fed rodents could identify additional preventative strategies against NASH. Here db/db mice, which spontaneously develop steatosis, were fed the MCD diet for 2 weeks in order to induce preeminent NASH. Lipid and methionine metabolism pathways were then characterized to identify the earliest steps in the transition from fatty liver to NASH. Comparisons were made with wild-type mice fed the MCD diet for 2 weeks in order to determine events induced by MCD feeding that require preexisting steatosis and thus may be indicative of the effects of synergism between methionine depletion and lipid accumulation. Ultimately, this study will provide new insights into the role of methionine and lipid metabolism in the sequential model of NASH development.

Methods

Animal Care

Three-month-old db/db B6 Cg-M^{+/+} female mice (related genotype, *a/a* + *Lepr^{db/+}* *Lepr^{db}*), a model of type 2 diabetes and the metabolic syndrome harboring a mutation in the leptin

receptor, and misty mice (related genotype, *a/a m +/m +*), expressing wild-type leptin receptors, were purchased from Jackson Laboratory (Bar Harbor, ME). They were fed ad libitum with either an MCD diet (MP Biomedicals Solon, OH, cat. no. 960439) as described elsewhere [7, 8, 23, 24] or the same diet supplemented with methionine and choline (i.e., control diet, MP Biomedicals cat. no. 960441) for 2 weeks. Each experimental group (two genotypes and two diets) consisted of five mice. Mice were housed individually in steel microisolator cages at 22°C with a 12-h/12-h, light/dark cycle. All procedures were conducted in accordance with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals and were approved by the Kansas University Medical Center institutional animal care and use committee. Blood samples were collected and centrifuged at 3,000g for 15 min. to collect serum. Livers were excised then snap-frozen in liquid nitrogen and kept at -70°C for RNA, protein, and lipid analysis. Separate liver slices were fixed in 10% formalin to be used for histology.

Histology

Formalin-fixed and paraffin-embedded liver sections were stained with hematoxylin-eosin for visual assessment of steatosis.

Serum Alanine Aminotransferase (ALT)

Serum ALT activity was determined using the Liquid ALT Reagent kit (Pointe Scientific Inc., Brussels, Belgium).

Triglycerides and Cholesterol

Total liver lipids were extracted from 50–100 mg of liver homogenate as previously described [25]. Hepatic triglyceride concentration was measured using a Triglyceride Test kit (Wako pure Chemical Industries, Richmond, VA), and hepatic cholesterol content was measured with a Cholesterol E-test kit (Wako pure Chemical Industries, Richmond, VA).

Thiobarbituric Acid Reactive Substances (TBARS)

Liver tissue (50 mg) was homogenized in 1.15% KCl. TBARS were quantified as malondialdehyde equivalents using the Zeptometrix OXItek TBARS assay kit (Zepto Metrix Corp. Buffalo, NY) modified to include 0.02% butylated hydroxytoluene during the boiling step to reduce ROS formation during boiling. TBARS were normalized to total protein in the homogenate as quantified by the Bradford method [26].

SAMe and S-Adenosylhomocysteine (SAH) Content

Liver tissue (45 mg) was homogenized in 1.15% KCl, then added to one volume of 10% trichloroacetic acid. The homogenate was then incubated on ice for 15 min, vortexed, and centrifuged at 10,000 rpm for 20 min. The supernatant was passed through a 0.45 µm filter and then applied to a high-performance liquid chromatography (HPLC) column. The levels of SAMe and SAH were measured as described previously [27].

GSH and Oxidized Glutathione (GSSG) Content

GSH and GSSG levels were measured using a Cayman chemical kit (Glutathione assay kit, Ann Arbor, MI, USA). Frozen liver tissue (50 mg) was homogenized in 2-(N-morpholino)ethanesulfonic acid (MES) buffer [0.4 M 2-(N-morpholino)ethanesulphonic acid, 0.1 M phosphate, and 2 mM ethylene diamine tetraacetic acid (EDTA), pH 6.0]. After centrifugation at 10,000g for 15 min, the supernatant was collected. For deproteinization, equal volumes of 10% metaphosphoric acid was added to the supernatant, incubated for 5 min, and spun at 2,000g for 2 min in a microcentrifuge. To 1 mL of the above supernatant, 50 μ L of 4 M triethanolamine (TEAM) was added to adjust the pH to 7.0, diluted, and assayed for GSH. To determine GSSG levels independent of GSH, 10 μ L of 2-vinylpyridine was added per milliliter of the diluted supernatant containing the TEAM reagent and incubated for 1 h at room temperature. Both GSH and GSSG were measured in these supernatants according to the manufacturer's protocol. Data was normalized to total protein in the homogenate as quantified by the Bradford method [26].

Quantitative Real-Time PCR

Livers were lysed in the TRIzol reagent (Invitrogen, Carlsbad, CA) and total RNA was isolated according to the manufacturer's protocol. RNA purity was confirmed by a 260/280 nm absorbance ratio greater than 1.5, and agarose gel electrophoresis with ethidium bromide staining was utilized to confirm the integrity of the 18S and 28S ribosomal RNA bands.

Reverse transcription was carried out using Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Primers and probes for real-time PCR amplification were designed using Primer Express 3.0 according to the manufacturer's instructions (Real-Time PCR Chemistry Guide, Applied Biosystems, Foster City, CA) and are listed in Table 2. All primer and probe sets were checked for specificity using the basic local alignment search tool (BLAST).

Real-time PCR amplification of cDNA corresponding to 16 ng of total RNA was carried out in a total volume of 20 μ L containing 1 $\times$ concentration of TaqMan[®] Universal Master Mix, 900 nM of each primer, and 150 nM of the 6-carboxyfluorescein (FAM)/6-carboxytetramethylrhodamine (TAMRA) dual-labeled probe. Amplification and fluorescence detection was carried out using the ABI Prism 7900 HT real time PCR system. Cycling conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s then 60°C for 1 min. Fourfold serial dilutions of reverse-transcribed RNA were amplified to create relative standard curves for quantification of each amplicon according to the manufacturer's instructions (Real-Time PCR Systems Chemistry Guide, Applied Biosystems, Foster City, CA). Gene expression values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Western Blotting

Frozen livers (100 mg) were homogenized in 1 mL of buffer containing 18 mM Tris, pH 7.5, 300 mM mannitol, 50 mM ethylene glycol tetraacetic acid (EGTA), and 0.1 mM phenylmethanesulfonyl fluoride at 4°C, followed by centrifugation at 10,000g for 20 min. The supernatant was collected and the protein content quantified using the Bradford method [26].

Cytosolic proteins (20 µg) were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels, electroblotted onto polyvinylidene difluoride membrane and immunoblotted with anti-MAT1/III and MATII antibodies (Santa Cruz Biotechnology, Inc. Santa Cruz, CA). Blots were blocked at 4°C with Tris-buffered saline with 0.1% Tween 20 (TBST) plus 5% dry nonfat milk. Blots were incubated in primary antibodies overnight at 4°C and washed three times in TBST. Blots were then incubated with the appropriate peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, INC. Santa Cruz, CA.) secondary antibodies diluted in TBST plus 5% milk for 1 h at room temperature. Following probing, blots were stripped and reprobed with GAPDH (ABcam Inc., Cambridge, MA). Proteins were viewed using enhanced chemiluminescence (PIERCE, Rockford, IL, USA). The intensities of the bands were quantified using Gel-Pro Analyzer 3.1 software.

Data Analysis

Values were compared using Student's two-tailed *t*-test. Significance was determined as $P < 0.05$.

Results

Body Weight

Wild-type mice fed the MCD diet exhibited significantly lower body weight than mice fed the control diet (Table 3). db/db mice fed either diet were significantly heavier than wild-type mice fed the same diet, and the difference between db/db mice fed the MCD diet and db/db mice fed the control diet did not reach statistical significance ($P = 0.070$).

Histology

Examination of liver histology revealed marked differences in liver tissue morphology before and after MCD diet feeding (Fig. 1). Wild-type mice fed the MCD diet developed mild microvesicular steatosis throughout the liver (Fig. 1b). Macrovesicular steatosis was observed in the livers of db/db mice fed the control diet (Fig. 1c). db/db mice fed the MCD diet exhibited severe macrovesicular steatosis with extensive hepatocyte ballooning (Fig. 1d).

Biochemistry

Serum triglyceride concentration was not different between db/db and wild-type mice fed the control diet (Table 3). After MCD diet feeding, serum triglycerides increased in both genotypes; however, the concentration was higher in db/db mice than in wild-type mice. Hepatic triglyceride content was higher in db/db mice than in wild-type mice when fed either diet. MCD diet feeding increased hepatic triglyceride content in db/db mice but did not significantly affect hepatic triglyceride content in wild-type mice. Serum cholesterol was elevated in db/db mice fed either diet in comparison with wild-type mice, and upon MCD diet feeding decreased in wild-type mice but was not affected in db/db mice. Hepatic cholesterol content was not different between genotypes or after MCD diet feeding. Basal serum ALT level in db/db mice was significantly higher (eightfold) compared to wild-type mice fed a control diet. Serum ALT increased significantly in wild type mice fed an MCD

diet but remained at much lower levels (14-fold difference after MCD diet feeding) than that of db/db mice fed the MCD diet. Feeding the db/db mice an MCD diet did not significantly change serum ALT levels compared to db/db mice fed the control diet; however, there was a trend towards an increase that nearly reached statistical significance ($P = 0.06$ compared to the db/db control). TBARS (a measure of reactive oxygen species and lipid peroxides, hereafter referred to collectively as reactive intermediates) were increased in wild-type mice fed an MCD diet. Basal levels of TBARS were higher in db/db mice fed a control diet when compared to wild-type mice. The MCD diet did not affect reactive intermediate production in db/db mice.

Inflammatory Gene Expression

Hepatic expression of TNF α mRNA was similar in both genotypes of mice fed a control diet (Fig. 2a). However, upon MCD diet feeding, TNF α mRNA level increased fourfold in db/db mice but was not induced in wild-type mice. The proinflammatory cytokine IL-1 β was only upregulated in db/db mice fed the MCD diet (Fig. 2b). Upregulation of the chemokine MIP-2 occurred after MCD diet feeding in db/db mice but was not induced in wild-type mice (Fig. 2c).

Fibrogenic Gene Expression

TGF β mRNA level was increased by MCD diet feeding in db/db mice but not wild-type mice; however, after MCD feeding, hepatic TGF β mRNA levels in db/db mice were not greater than that in wild-type mice (Fig. 3a). Level of α smooth-muscle actin (α -SMA) mRNA, which is a common biomarker of hepatic stellate cell activation, was lower in livers of db/db mice when compared to wild-type mice (Fig. 3b). Collagen 1 α mRNA level was not significantly changed by MCD diet feeding (Fig. 6c). Furthermore, Western blotting of α -SMA and morphological analysis confirmed that fibrosis was not present in any of the four groups (data not shown).

Lipid Synthesis and Storage Gene Expression

db/db mice expressed lower levels of fatty acid synthase (FAS; Fig. 4a) than wild-type mice when fed either diet. FAS mRNA level decreased in wild-type mice fed the MCD diet. Stearoyl-coA desaturase (SCD-1) mRNA was also expressed at lower basal levels in db/db mice than in wild-type mice (Fig. 4b), and in both genotypes, SCD-1 was markedly downregulated (12.5-fold and 13.5-fold in wild-type and db/db mice, respectively) by MCD diet feeding.

Lipid Oxidation Gene Expression

db/db mice expressed lower levels of PGC-1 α and PGC-1 β , the master regulators of energy metabolism, relative to wild-type mice when fed a control diet (Fig. 5a, b). PGC-1 α mRNA level increased after MCD diet feeding in db/db mice but remained lower than that of wild-type mice (Fig. 5a). MCD diet feeding increased PGC-1 β mRNA expression in db/db mice to a level comparable to that of wild-type mice fed either diet (Fig. 5b). db/db mice expressed CPT-1, the rate-limiting enzyme for mitochondrial β -oxidation, at a lower basal level than wild-type mice; however, after MCD diet feeding CPT-1 expression was similar

between genotypes due to reduced expression in wild-type mice (Fig. 5c). Cyp4a14 mRNA level was not significantly different between the two genotypes before MCD diet feeding (Fig. 5d); however, Cyp4a14 mRNA level increased after MCD diet feeding in db/db mice but not in wild-type mice.

Liver SAME and GSH Content

Liver SAME content was not different between genotypes in mice fed a control diet. However, MCD diet feeding reduced liver SAME content in db/db mice, but not in wild-type mice (Fig. 6a). Liver SAH content was neither affected by the MCD diet nor differed between genotypes (Fig. 6b). The SAME:SAH ratio was not different between genotypes fed a control diet. However, the SAME:SAH ratio was reduced by MCD feeding in db/db mice, but not in wild-type mice (Fig. 6c). GSH can be utilized to detoxify ROS via its oxidation to GSSG, and SAME is a precursor of GSH. Therefore, GSH utilization was measured as a function of GSH:GSSG ratio to determine if SAME depletion in db/db mice fed the MCD diet is a result of increased GSH utilization and subsequent replenishment. Steady-state hepatic content of the antioxidant GSH was not significantly affected by MCD diet feeding in either genotype; however, db/db mice fed the MCD diet had lower GSH content than control mice fed the same diet (Fig. 6d). Hepatic GSSG was also not affected by MCD diet feeding in either genotype (Fig. 6e). GSSG content was lower in db/db mice fed the MCD diet than in wild-type mice fed the same diet. Hepatic GSH:GSSG ratio was not significantly affected by MCD diet feeding in either genotype; however, GSH:GSSG ratio was higher in db/db mice fed the MCD diet compared to wild-type mice fed the same diet (Fig. 6f).

SAME Synthesis Pathway

The *MAT1A* and *MAT2A* genes encode the MATI/III and MATII enzymes, respectively, either of which are capable of synthesizing SAME from methionine and ATP. Basal MAT1A mRNA level was lower (threefold) in db/db mice than in wild-type mice (Fig. 7a). MAT1A mRNA expression increased during MCD diet feeding in both genotypes but remained lower in db/db mice relative to wild-type mice. Basal MAT2A mRNA expression was lower (five-fold) in db/db mice than in wild-type mice. MAT2A mRNA expression increased in db/db mice fed the MCD diet at a level twofold higher than that of wild-type mice, which did not significantly change during MCD diet feeding (Fig. 7b). MATI/III protein content was not different between genotypes and was not altered by MCD diet feeding (Fig. 7c). MATII protein content was not different between genotypes fed a control diet; however, upon MCD diet feeding, MATII protein abundance increased 19-fold in db/db mice but did not significantly change in wild-type mice (Fig. 7d). MATII protein abundance in db/db mice fed the MCD diet is not significantly different from the MATII protein level in wild-type mice fed a control diet, though the value approaches significance ($P = 0.056$). The MATII:MATI/III protein ratio was not different between genotypes of mice fed a control diet. However, MCD feeding induced a 13-fold increase of the MATII:MATI/III ratio in db/db mice but did not significantly alter this ratio in wild-type mice (Fig. 7e), resulting in an 11-fold higher MATII:MATI/III ratio in db/db mice fed the MCD diet relative to wild-type mice fed the same diet. MATII:MATI/III ratios were not significantly different between db/db mice fed the MCD diet and wild-type mice fed a control diet.

Discussion

The progression from fatty liver to NASH was investigated in db/db mice fed an MCD diet. MCD diet feeding is a common method of inducing steatosis, NASH, and fibrosis, presumably by inhibiting phosphatidylcholine synthesis and thus lipoprotein export from the liver [6, 7]. Whereas db/db mice fed the MCD diet for 4 weeks develop NASH and fibrosis [7] (Table 1), here the 2 week MCD feeding time point was characterized to identify potential causative biomarkers preceding NASH development. To this end, lipid and methionine metabolism pathways were characterized during early NASH development because (A) fatty liver precedes NASH in animal models [7, 8, reviewed in: 28] and (B) NASH induction by MCD feeding is dependent upon restriction of methionine but not choline [9].

Transition from Steatosis to NASH

NASH is characterized as a progression from benign steatosis to steatosis coupled with inflammation. db/db mice spontaneously develop severe steatosis associated with a moderate level of hepatocyte injury (serum ALT of 368 U/L), a level higher than described elsewhere [24]. Despite high serum ALT levels, db/db mice did not express elevated levels of proinflammatory cytokines. Upon 2 weeks of MCD diet feeding, levels of proinflammatory cytokines and the chemokine MIP-2 were increased in the livers of db/db mice. In contrast, mRNA levels of genes associated with fibrosis (i.e., α -smooth muscle actin, collagen 1 α) remained unchanged. Additionally, liver histology of db/db mice fed the MCD diet revealed increased hepatocyte ballooning but minimal inflammatory infiltrate. Thus, this treatment group represents early events in the transition from steatosis to NASH. Wild-type mice fed the MCD diet for 2 weeks were used to compare effects upon mice without preexisting fatty liver; therefore, biomarkers of NASH would not be expected in this group until later time points of MCD diet feeding (see Table 1).

Downregulation of Lipogenic Genes in db/db Mice and by MCD Diet Feeding

Diminished expression of the major lipogenic gene FAS suggests that steatosis in db/db mice fed a normal diet is not due to increased lipogenesis as suggested by others (Reviewed in: 28). Downregulation of SCD-1 in wild-type mice fed the MCD diet and low basal SCD-1 expression in db/db mice suggests that steatosis may be caused or maintained by inhibition of fatty acid incorporation into triglycerides for lipoprotein export, which has been suggested elsewhere [23].

Impaired Lipid Oxidation Pathway in db/db Mice

A possible cause of spontaneous lipid accumulation in the livers of db/db mice could be reduced hepatic lipid oxidation. Additionally, impaired lipid oxidation capacity could be a priming factor for NASH progression, whereas overloading of existing lipid oxidation pathways could result in upregulation of microsomal pathways and subsequent production of reactive intermediates [8].

db/db mice exhibited reduced mRNA expression of CPT-1, a rate-limiting enzyme in fatty acid oxidation responsible for import into mitochondria, when fed either a control or MCD

diet. Additionally, CPT-1 mRNA reduction also occurred in wild-type mice fed the MCD diet coincident with steatosis confirmed histologically. Thus, it is plausible that CPT-1 down-regulation contributes to the development of steatosis in both genotypes.

Additionally, PGC-1 α and PGC-1 β were expressed at low basal levels in db/db mice. PGC-1 α and PGC-1 β are central regulators of energy homeostasis that function by coactivating transcription factors involved in energy balance [29]. PGC-1 α knockout mice exhibit decreased mitochondrial number and respiratory capacity in skeletal muscle and hepatic steatosis [30]. PGC-1 β knockout mice are predisposed to liver steatosis when fed a high-fat diet corresponding to dysregulation of lipogenesis and reduced mitochondrial capacity [31]. Independent knockout of either PGC-1 α or PGC-1 β is sufficient to induce major disruptions of energy homeostasis, suggesting that these two genes are not functionally redundant. Consistent with the current study, Aoyama also observed decreased PGC-1 α mRNA levels in the livers of fasting db/db mice [32]. In the current study, PGC-1 β was not different between genotypes after MCD diet feeding, whereas PGC-1 α remained lower in db/db mice relative to wild-type mice; thus, induction of PGC-1 β was not sufficient to rescue steatosis or prevent NASH progression.

Cyp4a14 is involved in microsomal ω -oxidation and is upregulated when the mitochondrial and peroxisomal lipid oxidation pathways are overloaded or inhibited [reviewed in 33]. ω -oxidation generates toxic dicarboxylic fatty acids as well as hydrogen peroxide, which could result in cellular insult. db/db mice fed the MCD diet upregulated Cyp4a14 mRNA, suggesting that other lipid oxidation pathways are overloaded during MCD diet feeding. Enrichment of microsomal ω -oxidation has been described elsewhere [8] in wild-type mice fed the MCD diet for an extended period of time and here we identify this common biomarker in db/db mice progressing to NASH.

db/db mice produced elevated basal reactive intermediates relative to wild-type mice; however, reactive intermediates were not further induced by MCD feeding in db/db mice. Thus, production of reactive intermediates at this level is not sufficient to induce NASH. It is important to note that reactive intermediate production preceded Cyp4a14 upregulation, suggesting that pathways independent of Cyp4a14 contributed to reactive intermediate production in db/db mice. Production of reactive intermediates by wild-type mice fed the MCD diet for an extended duration has been described elsewhere [8, 24] and thus further induction may be expected after extended MCD feeding of db/db mice. Despite producing elevated basal reactive intermediates, db/db mice did not exhibit increased utilization of the antioxidant GSH. Again, GSH depletion and increased GSH utilization at later time-points [9, 11] has been described and would be expected with extended MCD feeding.

SAMe Depletion in Transition from NAFLD to NASH

Here we observed depleted SAMe levels and reduced SAMe:SAH ratios in the livers of db/db mice fed an MCD diet for 2 weeks. This phenomenon was unique to this treatment group, which is the only group exhibiting early NASH development. SAMe is the principal biological methyl donor required for lipoprotein formation (and thus export) and GSH synthesis. Additionally, SAMe methylation pathways influence proliferation, apoptosis, differentiation, DNA synthesis, membrane fluidity, and ROS generation [10]. SAMe is

synthesized from methionine and ATP by MAT enzymes, which are present in two main isoforms, MATI/III, the liver-specific MAT enzymes consisting of subunits encoded by the *MAT1A* gene, or MAT II, the non-liver-specific MAT isoform encoded by the *MAT2A* gene [10]. MATII is inhibited by high levels of SAmE; thus, MATI/III enzymes are capable of maintaining higher tissue SAmE concentrations.

SAmE depletion could result from reduced synthesis or increased utilization. In the current study, SAmE reduction was associated with upregulation of the non-liver-specific MAT2A mRNA and protein (MATII) in db/db mice fed the MCD diet. This upregulation resulted in increased MATII:MATI/III protein ratios, which may be responsible for reduced SAmE synthesis in livers of db/db mice fed the MCD diet. The MATII protein was not up-regulated in wild-type mice fed the MCD diet, and SAmE levels did not fall in this treatment group, suggesting a role for an increased MATII:MATI/III ratio in decreased hepatic SAmE content. However, neither the MATII protein level nor the MATII:MATI/III ratio were significantly different between db/db mice fed the MCD diet and wild-type mice fed a control diet, although the difference in MATII protein level approached a significant value ($P = 0.056$). This finding demonstrates that increased MATII:MATI/III ratio is not sufficient to induce SAmE depletion, suggesting that other factors unique to db/db mice have additive or synergistic effects with differential MAT expression to contribute to SAmE depletion induced by the MCD diet. Elevated MATII:MATI/III ratio and reduced hepatic SAmE levels have been implicated in a number of pathological liver conditions such as thioacetamide [14], CCL₄ [20], and ethanol treatment [16] and have been associated with proliferating livers (HCC) [34] and regenerating livers [35]. Additionally, *MAT1A* knockout mice exhibit low SAmE levels in the liver and spontaneously develop NASH [36]. Finally, wild-type rats fed the MCD diet until the onset of fibrosis also demonstrate reduced SAmE levels [9]. Accordingly, it would be expected that wild-type mice fed the MCD diet would eventually exhibit SAmE depletion after fatty liver is established.

Increased SAmE utilization likely contributes to SAmE depletion; however, increased utilization of GSH, for which SAmE is a precursor, did not occur in mice with SAmE depletion. Thus, increased SAmE utilization via other methylation reactions, perhaps induced by altered lipid metabolism pathways in fatty livers, are likely the cause of SAmE depletion of db/db mice. Identification of these pathways merits further investigation.

If SAmE depletion indeed contributes to the second hit of NASH development, dietary SAmE supplementation may be an effective therapy for prevention of the transition from fatty liver to NASH. In rats fed the MCD diet, SAmE administration beginning at the onset of fibrosis has been shown to attenuate further liver injury [11]. In other animal studies, SAmE supplementation improves a variety of pharmacologically induced liver injuries such as alcohol-induced steatohepatitis [13], fibrosis induced by CCL₄ [19] or thioacetamide [20], HCC induced by diethylnitrosamine/phenobarbital treatment [17], and acute necrosis induced by Acetaminophen [12] or D-galactosamine [18]. Oral administration of SAmE in humans significantly increases GSH levels in patients with alcoholic cirrhosis or nonalcoholic liver disease [37] and also reduces mortality and delays liver transplantation in patients with alcoholic cirrhosis [38]. The association of SAmE depletion and differential MAT expression specifically with early-stage NASH is a novel finding that supports the

potential for SAME supplementation as a preventative measure against NASH in patients with fatty liver.

Summary

The MCD diet is a classical method of inducing fatty liver and NASH. Because NASH induction by the MCD diet requires lipid accumulation and methionine restriction, abnormalities of lipid and methionine metabolism were identified in db/db mice in the transition from fatty liver to NASH. Inhibited lipid oxidation was found to be a common characteristic of mice with steatosis and thus may serve as a priming factor for lipid accumulation and eventual NASH induction. Depletion of the methionine metabolite SAME occurred in mice progressing to NASH but not in mice with steatosis only. SAME depletion exerts pleiotropic effects upon liver homeostasis and thus may set into motion the insults leading to NASH. This finding supports a therapeutic potential for SAME supplementation in individuals with fatty liver who, in turn, exhibit a high risk of developing NASH.

Acknowledgments

This study is supported by NIH grants CA53596 and AA14147, and COBRE P20 RR021940 as well as the Molecular Biology Core supported by the COBRE grant.

References

- Farrell GC, Larter CZ. Nonalcoholic fatty liver disease: From steatosis to cirrhosis. *Hepatology*. 2006; 43:S99–S112. [PubMed: 16447287]
- Wanless IR, Lentz JS. Fatty liver hepatitis (steatohepatitis) and obesity: An autopsy study with analysis of risk factors. *Hepatology*. 1990; 12:1106–1110. [PubMed: 2227807]
- Brunt EM. Nonalcoholic steatohepatitis. *Semin Liver Dis*. 2004; 24:3–20. [PubMed: 15085483]
- Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor- α : Direct role in obesity-linked insulin resistance. *Science*. 1993; 259:87–91. [PubMed: 7678183]
- Lee GH, Proenca R, Montez JM, Carroll KM, Darvishzadeh JG, Lee JJ, Friedman JM. Abnormal splicing of the leptin receptor in diabetic mice. *Nature*. 1996; 379:632–635. [PubMed: 8628397]
- Pascale R, Pirisi L, Daino L, Zanetti S, Satta A, Bartoli E, Feo F. Role of phosphatidylethanolamine methylation in the synthesis of phosphatidylcholine by hepatocytes isolated from choline-deficient rats. *FEBS Lett*. 1982; 145:293–297. [PubMed: 7128827]
- Sahai A, Malladi P, Pan X, Paul R, Melin-Aldana H, Green RM, Whittington PF. Obese and diabetic db/db mice develop marked liver fibrosis in a model of nonalcoholic steatohepatitis: Role of short-form leptin receptors and osteopontin. *Am J Physiol Gastrointest Liver Physiol*. 2004; 287:G1035–G1043. [PubMed: 15256362]
- Leclercq IA, Farrell GC, Field J, Bell DR, Gonzalez FJ, Robertson GR. Cyp2e1 and cyp4a as microsomal catalysts of lipid peroxides in murine nonalcoholic steatohepatitis. *J Clin Invest*. 2000; 105:1067–1075. [PubMed: 10772651]
- Oz HS, Chen TS, Neuman M. Methionine deficiency and hepatic injury in a dietary steatohepatitis model. *Dig Dis Sci*. 2007 Aug 21. (Epub ahead of print).
- Mato JM, Lu SC. Role of S-adenosyl-L-methionine in liver health and injury. *Hepatology*. 2007; 45:1306–1312. [PubMed: 17464973]
- Oz HS, Im HJ, Chen TS, de Villiers WJ, McClain CJ. Glutathione-enhancing agents protect against steatohepatitis in a dietary model. *J Biochem Mol Toxicol*. 2006; 20:39–47. [PubMed: 16498637]
- Bray GP, Tredger JM, Williams R. S-adenosylmethionine protects against acetaminophen hepatotoxicity in two mouse models. *Hepatology*. 1992; 15:297–301. [PubMed: 1735533]

13. Garcia-Ruiz C, Morales A, Colell A, Ballesta A, Rodes J, Kaplowitz N, Fernandez-Checa JC. Feeding s-adenosyl-l-methionine attenuates both ethanol-induced depletion of mitochondrial glutathione and mitochondrial dysfunction in periportal and perivenous rat hepatocytes. *Hepatology*. 1995; 21:207–214. [PubMed: 7806156]
14. Huang ZZ, Mato JM, Kanel G, Lu SC. Differential effect of thioacetamide on hepatic methionine adenosyltransferase expression in the rat. *Hepatology*. 1999; 29:1471–1478. [PubMed: 10216131]
15. Lieber CS, Casini A, DeCarli LM, Kim CI, Lowe N, Sasaki R, Leo MA. S-adenosyl-l-methionine attenuates alcohol-induced liver injury in the baboon. *Hepatology*. 1990; 11:165–172. [PubMed: 2307395]
16. Lu SC, Huang ZZ, Yang H, Mato JM, Avila MA, Tsukamoto H. Changes in methionine adenosyltransferase and s-adenosylmethionine homeostasis in alcoholic rat liver. *Am J Physiol Gastrointest Liver Physiol*. 2000; 279:G178–G185. [PubMed: 10898761]
17. Pascale RM, Marras V, Simile MM, Daino L, Pinna G, Bennati S, Carta M, Seddaiu MA, Massarelli G, Feo F. Chemoprevention of rat liver carcinogenesis by s-adenosyl-l-methionine: a long-term study. *Cancer Res*. 1992; 52:4979–4986. [PubMed: 1516054]
18. Stramentinoli G, Gualano M, Ideo G. Protective role of s-adenosyl-l-methionine on liver injury induced by d-galactosamine in rats. *Biochem Pharmacol*. 1978; 27:1431–1433. [PubMed: 697884]
19. Varela-Moreiras G, Alonso-Aperte E, Rubio M, Gasso M, Deulofeu R, Alvarez L, Caballeria J, Rodes J, Mato JM. Carbon tetrachloride-induced hepatic injury is associated with global DNA hypomethylation and homocysteinemia: Effect of s-adenosylmethionine treatment. *Hepatology*. 1995; 22:1310–1315. [PubMed: 7557886]
20. Mato JM, Alvarez L, Ortiz P, Pajares MA. S-adenosylmethionine synthesis: molecular mechanisms and clinical implications. *Pharmacol Ther*. 1997; 73:265–280. [PubMed: 9175157]
21. Duce AM, Ortiz P, Cabrero C, Mato JM. S-adenosyl-l-methionine synthetase and phospholipid methyltransferase are inhibited in human cirrhosis. *Hepatology*. 1988; 8:65–68. [PubMed: 3338721]
22. Avila MA, Berasain C, Torres L, Martin-Duce A, Corrales FJ, Yang H, Prieto J, Lu SC, Caballeria J, Rodes J, Mato JM. Reduced mrna abundance of the main enzymes involved in methionine metabolism in human liver cirrhosis and hepatocellular carcinoma. *J Hepatol*. 2000; 33:907–914. [PubMed: 11131452]
23. Rizki G, Arnaboldi L, Gabrielli B, Yan J, Lee GS, Ng RK, Turner SM, Badger TM, Pitas RE, Maher JJ. Mice fed a lipogenic methionine-choline-deficient diet develop hypermetabolism coincident with hepatic suppression of *scd-1*. *J Lipid Res*. 2006; 47:2280–2290. [PubMed: 16829692]
24. Yamaguchi K, Yang L, McCall S, Huang J, Yu XX, Pandey SK, Bhanot S, Monia BP, Li YX, Diehl AM. Inhibiting triglyceride synthesis improves hepatic steatosis but exacerbates liver damage and fibrosis in obese mice with nonalcoholic steatohepatitis. *Hepatology*. 2007; 45:1366–1374. [PubMed: 17476695]
25. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem*. 1957; 226:497–509. [PubMed: 13428781]
26. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976; 72:248–254. [PubMed: 942051]
27. She QB, Nagao I, Hayakawa T, Tsuge H. A simple hplc method for the determination of s-adenosylmethionine and s-adenosylhomocysteine in rat tissues: the effect of vitamin b6 deficiency on these concentrations in rat liver. *Biochem Biophys Res Commun*. 1994; 205:1748–1754. [PubMed: 7811261]
28. Koteish A, Mae Diehl A. Animal models of steatohepatitis. *Best Pract Res Clin Gastroenterol*. 2002; 16:679–690. [PubMed: 12406439]
29. Finck BN, Kelly DP. Pgc-1 coactivators: inducible regulators of energy metabolism in health and disease. *J Clin Invest*. 2006; 116:615–622. [PubMed: 16511594]
30. Leone TC, Lehman JJ, Finck BN, Schaeffer PJ, Wende AR, Boudina S, Courtois M, Wozniak DF, Sambandam N, Bernal-Mizrachi C, Chen Z, Holloszy JO, Medeiros DM, Schmidt RE, Saffitz JE, Abel ED, Semenkovich CF, Kelly DP. Pgc-1alpha deficiency causes multi-system energy

metabolic derangements: muscle dysfunction, abnormal weight control and hepatic steatosis. *PLoS Biol.* 2005; 3:e101. [PubMed: 15760270]

31. Sonoda J, Mehl IR, Chong LW, Nofsinger RR, Evans RM. Pgc-1beta controls mitochondrial metabolism to modulate circadian activity, adaptive thermogenesis, and hepatic steatosis. *Proc Natl Acad Sci USA.* 2007; 104:5223–5228. [PubMed: 17360356]
32. Aoyama H, Daitoku H, Fukamizu A. Nutrient control of phosphorylation and translocation of foxo1 in c57bl/6 and db/db mice. *Int J Mol Med.* 2006; 18:433–439. [PubMed: 16865227]
33. Koteish A, Diehl AM. Animal models of steatosis. *Semin Liver Dis.* 2001; 21:89–104. [PubMed: 11296700]
34. Cai J, Sun WM, Hwang JJ, Stain SC, Lu SC. Changes in s-adenosylmethionine synthetase in human liver cancer: Molecular characterization and significance. *Hepatology.* 1996; 24:1090–1097. [PubMed: 8903381]
35. Huang ZZ, Mao Z, Cai J, Lu SC. Changes in methionine adenosyltransferase during liver regeneration in the rat. *Am J Physiol.* 1998; 275:G14–G21. [PubMed: 9655679]
36. Lu SC, Alvarez L, Huang ZZ, Chen L, An W, Corrales FJ, Avila MA, Kanel G, Mato JM. Methionine adenosyltransferase 1a knockout mice are predisposed to liver injury and exhibit increased expression of genes involved in proliferation. *Proc Natl Acad Sci USA.* 2001; 98:5560–5565. [PubMed: 11320206]
37. Vendemiale G, Altomare E, Trizio T, Le Grazie C, Di Padova C, Salerno MT, Carrieri V, Albano O. Effects of oral s-adenosyl-l-methionine on hepatic glutathione in patients with liver disease. *Scand J Gastroenterol.* 1989; 24:407–415. [PubMed: 2781235]
38. Mato JM, Camara J, Fernandez de Paz J, Caballeria L, Coll S, Caballero A, Garcia-Buey L, Beltran J, Benita V, Caballeria J, Sola R, Moreno-Otero R, Barrao F, Martin-Duce A, Correa JA, Pares A, Barrao E, Garcia-Magaz I, Puerta JL, Moreno J, Boissard G, Ortiz P, Rodes J. S-adenosylmethionine in alcoholic liver cirrhosis: A randomized, placebo-controlled, double-blind, multi-center clinical trial. *J Hepatol.* 1999; 30:1081–1089. [PubMed: 10406187]

Fig. 1. Liver histology of wild-type and db/db mice fed the MCD diet. Wild-type (a and b) or db/db (c and d) mice were fed either control diets (a and c) or the MCD diet for 2 weeks (b and d). Liver sections were stained with hematoxylin-eosin to assess morphology. Micrographs are representative of five mice of each group

Fig. 2.

Hepatic TNF α , IL-1 β , and MIP-2 mRNA levels in wild-type and db/db mice fed the MCD diet. Wild-type (□) or db/db (■) mice were fed either a control diet or the MCD diet for 2 weeks. mRNA levels of (a) TNF α , (b) IL-1 β , and (c) MIP-2 was quantified relative to GAPDH as described in the Materials and Methods section. Data represent mean $\pm$ SD ($n = 5$). * $P < 0.05$ compared to wild-type mice fed the same diet, # $P < 0.05$ compared to control diet-fed mice of the corresponding genotype

Fig. 3.

Hepatic TGFβ, α-SMA, and collagen 1α mRNA levels in wild-type and db/db mice fed the MCD diet. Wild-type (□) or db/db (■) mice were fed either a control diet or the MCD diet for 2 weeks. mRNA levels of (a) TGFβ, (b) α-SMA, and (c) collagen 1α was quantified relative to GAPDH as described in the Materials and Methods section. Data represent mean ± SD (*n* = 5). **P* < 0.05 compared to wild-type mice fed the same diet, #*P* < 0.05 compared to control-diet-fed mice of the corresponding genotype

Fig. 4.

Hepatic FAS and SCD-1 mRNA levels in wild-type and db/db mice fed the MCD diet. Wild-type (□) or db/db (■) mice were fed either a control diet or the MCD diet for 2 weeks. mRNA levels of (a) FAS and (b) SCD-1 was quantified relative to GAPDH as described in the Materials and Methods section. Data represent mean $\pm$ SD ($n = 5$). * $P < 0.05$ compared to wild-type mice fed the same diet, # $P < 0.05$ compared to control-diet-fed mice of the corresponding genotype

Fig. 5.

Hepatic PGC-1 α , PGC-1 β , CPT-1, and Cyp4a14 mRNA levels in wild-type and db/db mice fed the MCD diet. Wild-type (□) or db/db (■) mice were fed either a control diet or the MCD diet for 2 weeks. mRNA levels of (a) PGC-1 α , (b) PGC-1 β , (c) CPT-1, and (d) Cyp4a14 expression was quantified relative to GAPDH as described in the Materials and Methods section. Data represent mean $\pm$ SD ($n = 5$). * $P < 0.05$ compared to wild-type mice fed the same diet, # $P < 0.05$ compared to control-diet-fed mice of the corresponding genotype

Fig. 6. Hepatic SAME and GSH metabolism of wild-type and db/db mice fed the MCD diet. Wild-type (□) or db/db (■) mice were fed either a control diet or the MCD diet for 2 weeks. Hepatic (a) SAME concentrations, (b) SAH concentrations, (c) SAME:SAH ratios, (d) GSH concentrations, (e) GSSG concentrations, and (f) GSH:GSSG ratios were quantified as described in the Materials and Methods section. Data represent mean $\pm$ SD (a, b, and c, $n = 3$; d, e, and f, $n = 5$). * $P < 0.05$ compared to wild-type mice fed the same diet, # $P < 0.005$ compared to control-diet-fed mice of the corresponding genotype

Fig. 7.

Hepatic MATI/III and MATII mRNA and protein levels of wild-type and db/db mice fed the MCD diet. Wild-type (□) or db/db (■) mice were fed either a control diet or the MCD diet for 2 weeks. mRNA levels of (a) MAT1A and (b) MAT2A were quantified relative to GAPDH as described in the Materials and Methods section. (c) MATI/III and (d) MATII protein levels and (e) MATII:MATI/III ratio was quantified relative to GAPDH using Western blotting as described in the Materials and Methods section. Densitometry graphs (c) and (d) are accompanied with representative blots chosen from three samples per group.

Data represent mean $\pm$ SD (**a** and **b**, $n = 5$; **c**, **d**, and **e**, $n = 3$). * $P < 0.05$ compared to wild-type mice fed the same diet, # $P < 0.05$ compared to control-diet-fed mice of the corresponding genotype

Table 1

Time line of disease progression in wild-type or db/db mice fed the MCD diet

	0	2W	4W	8W	10W
Wild type					
Steatosis	-		+ (c.s.)	+++ [7]	n.d.
Inflamm.	-		-(c.s.)	++ [7]	n.d.
Fibrosis	-		-(c.s.)	-[7]	n.d.
db/db					
Steatosis	+++ (c.s.)		+++ (c.s.)	+++ [7]	++ [24]
Inflamm.	-(c.s.)		+ (c.s.)	+++ [7]	+++ [24]
Fibrosis	-(c.s.)		-(c.s.)	+++ [7]	+++ [24]

Qualitative data are summarized from [7], [8], [24], and the current study (c.s.). Timepoint zero represents mice fed a control diet. Timeline is not drawn to scale. Symbols represent: -, not present; +, mild; ++, moderate; +++, severe; n.d., no data available. Inflamm., inflammation

Table 2

Sequences of primers and FAM-TAMRA-labeled TaqMan probes used for quantitative real-time PCR

Gene	Oligo	Sequence	Accession no.
Collagen 1 α	Sense	CCCGCCGATGTCGCTAT	NM_007742
	Antisense	GCTACGCTGTTCTTGCACTGAT	
	Probe	TTCCTGCGCCTAATGTCCACCGA	
Carnitine palmitoyltransferase 1 (CPT-1)	Sense	CGATCATCATGACTATGCGCTACT	NM_000085.5
	Antisense	GCCGTGCTCTGCAAACATC	
	Probe	CTGAAGGTGCTGCTCTCCTACCATTCA	
Cytochrome P450 4a14 (Cyp4a14)	Sense	CAAGACCCTCCAGCATTTCC	NM_007822
	Antisense	GAGCTCCTTGTCCTTCAGATGGT	
	Probe	TGCATGCCTTCCCACTGGCTTTG	
Fatty acid synthase (FAS)	Sense	CCCGGAGTCGCTTGAGTATATT	NM_007988.3
	Antisense	GGACCGAGTAATGCCATTGAG	
	Probe	AGCCCATGGCACGGGCACC	
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Sense	TGTGTCCGTCGTGGATCTGA	NM_001001303
	Antisense	CCTGCTTACCACCTTCTTGA	
	Probe	CCGCCTGGAGAAACCTGCCA	
Interleukin 1 β (IL-1 β)	Sense	AAGATGAAGGGCTGCTTCCA	NM_008361
	Antisense	GTGCTGCTGCGAGATTGAA	
	Probe	CCTTTGACCTGGGCTGTCCTGATGA	
Methionine adenosyltransferase 1A (MAT1A)	Sense	TGATGAGACCGAGGAATGCA	NM_133653.2
	Antisense	GATCTGCTATCCGGGTGTTGAG	
	Probe	CCCTTACCATCGTGCTCGCTCACAA	
Methionine adenosyltransferase 2A (MAT2A)	Sense	CACAAGCTAAATGCCAAATTGG	NM_145569.3
	Antisense	TTGAGTTTTAGAATCTGGGCGTAA	
	Probe	TGAACTACGCCGAATGGTACATTGC	
Macrophage inflammatory protein 2 (MIP-2)	Sense	GAACATCCAGAGCTTGAGTGTGA	NM_009140
	Antisense	CCCTTGAGAGTGGCTATGACTTC	
	Probe	AGGACCCCACTGCGCCGAGA	
PPAR γ Coactivator 1 α (PGC-1 α)	Sense	AACCACACCCACAGGATCAGA	NM_008904
	Antisense	TCTTCGCTTTATTGCTCCATGA	
	Probe	CAAACCCTGCCATTGTTAAGACCGAGAA	
PPAR γ Coactivator 1 β (PGC-1 β)	Sense	TGACTCAGCCACGTGCTTTG	NM_133249.2
	Antisense	TCGTAAGCGCAGCCAAGAG	
	Probe	CGAGCTCTTCCAGATTGACAGTGAGAATGA	
Stearoyl-CoA desaturase 1 (SCD-1)	Sense	CGTTCCAGAATGACGTGTACGA	NM_009127.3
	Antisense	AGGGTCGGCGTGTGTTTC	
	Probe	CACCGCGCCCAACACAAGTTCT	
α -Smooth Muscle Actin (α -SMA)	Sense	CCTGACGGGCAGGTGATC	NM_007392
	Antisense	ATGAAAGATGGCTGGAAGAGAGTCT	
	Probe	CGAACGCTTCCGCTGCCCA	

Gene	Oligo	Sequence	Accession no.
Tumor growth factor β (TGF β)	Sense	GAGCCCGAAGCGGACTACTA	NM_011577
	Antisense	GTTTTCTCATAGATGGCGTTGTTG	
	Probe	CACCCGCGTGCTAATGGTGGACC	
Tumor necrosis factor α (TNF α)	Sense	ACAAGGCTGCCCCGACTAC	NM_013693
	Antisense	TTTCTCCTGGTATGAGATAGCAAATC	
	Probe	TGCTCCTCACCCACACCGTCAGC	

Table 3

Liver and serum biochemical parameters in wild-type and db/db mice fed the MCD diet

	WT CTRL	WT MCD	db/db CTRL	db/db MCD
Body weight (g)	20.1 ± 0.5	15.5 ± 1.2 [#]	47.1 ± 0.9 [*]	41.9 ± 5.5 [*]
Liver triglycerides (mg/mg protein)	110 ± 17	135 ± 31	183 ± 35 [*]	278 ± 48 ^{#, *}
Serum triglycerides (mg/dL)	19.6 ± 3.3	32.3 ± 11 [#]	24.5 ± 7.0	63.0 ± 7.8 ^{#, *}
Liver cholesterol (mg/mg protein)	15.7 ± 3.3	16.8 ± 3.2	17.0 ± 3.2	18.4 ± 2.5
Serum cholesterol (mg/dL)	61.1 ± 6.9	42.8 ± 14 [#]	157 ± 23 [*]	124 ± 27 [*]
Serum ALT (U/L)	45.0 ± 9.6	78.8 ± 28 [#]	368 ± 150 [*]	1110 ± 620 [*]
TBARS (nmol/mg protein)	34.3 ± 3.2	54.3 ± 2.9 [#]	62.3 ± 10 [*]	49 ± 4.5

Triglycerides, cholesterol, ALT, and TBARS were determined as described in the Materials and Methods section. TBARS are expressed as malondialdehyde equivalents, representing ROS and reactive aldehydes. Data are presented as means ± standard deviation (SD)

[#] Significantly different from mice of the same genotype fed a control diet ($P < 0.05$)

^{*} Significantly different from wild-type mice fed the same diet ($P < 0.05$)